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(54) Title: IMMUNOGENS THAT INDUCE CYTOTOXIC T-LYMPHOCYTES AND THEIR USE IN PREVENTION, TREATMENT, AND DIAGNOSIS OF CANCER

(57) Abstract: The present invention relates to compositions and methods for the prevention, treatment, and diagnosis of cancer, especially carcinomas, such as breast and ovarian carcinoma. The invention discloses peptides, polypeptides, and polynucleotides that can be used to stimulate a cellular immune response against breast or ovarian cancer.



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# IMMUNOGENS THAT INDUCE CYTOTOXIC T-LYMPHOCYTES AND THEIR USE IN PREVENTION, TREATMENT, AND DIAGNOSIS OF CANCER

## Cross Reference to Related Applications

This application is based on and claims priority to U.S. Provisional Application No. 60/993,775, the disclosure of which is herein incorporated by reference.

## Field of the Invention

The present invention relates generally to the field of immunogens whose structures incorporate polypeptides comprising epitopic peptides derived from proteins expressed by cancer cells and to uses of said immunogens in eliciting cytotoxic T lymphocyte (CTL) responses for the diagnosis, prevention and treatment of cancer, preferably carcinoma, most preferably breast or ovarian carcinoma.

## Background of the Invention

The mammalian immune system has evolved a variety of mechanisms to protect the host from cancerous cells, an important component of this response being mediated by cells referred to as T cells. Cytotoxic T lymphocytes (CTLs) are specialized T cells that function primarily by recognizing and killing cancerous cells or infected cells, but also by secreting soluble molecules referred to as cytokines that can mediate a variety of effects on the immune system.

Evidence suggests that immunotherapy designed to stimulate a tumor-specific CTL response would be effective in controlling cancer. For example, it has been shown that human CTLs recognize sarcomas (Slovin, S. F. et al., J. Immunol., 137:3042-3048, (1987)), renal cell carcinomas (Schendel, D. J. et al., J. Immunol., 151:4209-4220, (1993)), colorectal carcinomas (Jacob, L. et al., Int. J. Cancer, 71:325-332, (1997)), ovarian carcinomas (Ioannides, C. G. et al., J. Immunol., 146:1700-1707, (1991)) (Peoples, G. E. et al., Surgery, 114:227-234, (1993)), pancreatic carcinomas (Peiper, M. et al., Eur.J.Immunol., 27:1115-1123, (1997); Wolfel, T. et al., Int.J.Cancer, 54:636-644, (1993)), squamous tumors of the head and neck (Yasumura, S. et al., Cancer Res., 53:1461-1468, (1993)), and squamous carcinomas of the lung (Slingluff, C. L. Jr et al., Cancer Res., 54:2731-2737, (1994); Yoshino, I. et al., Cancer Res., 54:3387-3390, (1994)). The largest number of reports of human tumor-reactive CTLs have concerned cancers (Boon, T. et al., Ann.Rev.Immunol., 12:337-365, (1994)). The ability of tumor-specific CTLs to mediate tumor regression, in both

human (Rosenberg, S. A. et al., N.Engl.J.Med., 319:1676-1680, (1988)) and animal models (Celluzzi, C. M. et al., J.Exp.Med., 183:283-287, (1996); Mayordomo, J. I. et al., Nat.Med., 1:1297-1302, (1995); Zitvogel, L. et al., J.Exp.Med., 183:87-97, (1996)), suggests that methods directed at increasing CTL activity would likely have a beneficial effect with respect to tumor treatment.

In order for CTLs to kill or secrete cytokines in response to a cancer cell, the CTL must first recognize that cell as being cancerous. This process involves the interaction of the T cell receptor, located on the surface of the CTL, with what is generically referred to as an MHC-peptide complex which is located on the surface of the cancerous cell. MHC (Major Histocompatibility Complex)-encoded molecules have been subdivided into two types, and are referred to as class I and class II MHC-encoded molecules.

In the human immune system, MHC molecules are referred to as human leukocyte antigens (HLA). Within the MHC, located on chromosome six, are three different genetic loci that encode for class I MHC molecules. MHC molecules encoded at these loci are referred to as HLA-A, HLA-B, and HLA-C. The genes that can be encoded at each of these loci are extremely polymorphic, and thus, different individuals within the population express different class I MHC molecules on the surface of their cells. HLA-A1, HLA-A2, HLA-A3, HLA-B7, and HLA-B8 are examples of different class I MHC molecules that can be expressed from these loci. The present disclosure involves peptides that are associated with the HLA-A1, HLA-A2, or HLA-A3 molecules, HLA-A1 supertypes, HLA-A2 supertypes, and HLA-A3 supertypes. A supertype is a group of HLA molecules that present at least one shared epitope. The present disclosure involves peptides that are associated with HLA molecules, and with the genes and proteins from which these peptides are derived.

The peptides that associate with the MHC molecules can either be derived from proteins made within the cell, in which case they typically associate with class I MHC molecules (Rock, K. L. and Golde, U., Ann. Rev. Immunol., 17:739-779, (1999)) or they can be derived from proteins that are acquired from outside of the cell, in which case they typically associate with class II MHC molecules (Watts, C., Ann. Rev. Immunol., 15:821-850, (1997)). Peptides that evoke a cancer-specific CTL response most typically associate with class I MHC molecules. The peptides that associate with a class I MHC molecule are typically nine amino acids in length, but can vary from a minimum length of eight amino acids to a maximum of fourteen amino acids in length. A class I MHC molecule with its

bound peptide, or a class II MHC molecule with its bound peptide, is referred to as an MHC-peptide complex.

The process by which intact proteins are degraded into peptides is referred to as antigen processing. Two major pathways of antigen processing occur within cells (Rock, K. L. and Golde, U., *Ann.Rev.Immunol.*, 17:739-779, (1999); Watts, C., *Ann. Rev. Immunol.*, 15:821-850, (1997)). One pathway, which is largely restricted to cells that are antigen presenting cells such as dendritic cells, macrophages, and B cells, degrades proteins that are typically phagocytosed or endocytosed into the cell. Peptides derived in this pathway typically bind to class II MHC molecules. A second pathway of antigen processing is present in essentially all cells of the body. This second pathway primarily degrades proteins that are made within the cells, and the peptides derived from this pathway primarily bind to class I MHC molecules. It is the peptides from this second pathway of antigen processing that are referred to herein. Antigen processing by this latter pathway involves polypeptide synthesis and proteolysis in the cytoplasm. The peptides produced are then transported into the endoplasmic reticulum of the cell, associate with newly synthesized class I MHC molecules, and the resulting MHC-peptide complexes are then transported to the cell surface. Peptides derived from membrane and secreted proteins may also associate with Class I MHC molecules. In some cases these peptides correspond to the signal sequence of the proteins that are cleaved from the protein by the signal peptidase. In other cases, it is thought that some fraction of the membrane and secreted proteins are transported from the endoplasmic reticulum into the cytoplasm where processing subsequently occurs.

Once bound to the class I MHC molecule and displayed on the surface of a cell, the peptides are recognized by antigen-specific receptors on CTLs. Mere expression of the class I MHC molecule itself is insufficient to trigger the CTL to kill the target cell if the antigenic peptide is not bound to the class I MHC molecule. Several methods have been developed to identify the peptides recognized by CTL, each method relying on the ability of a CTL to recognize and kill only those cells expressing the appropriate class I MHC molecule with the peptide bound to it (Rosenberg, S. A., *Immunity*, 10:281-287, (1999)). Such peptides can be derived from a non-self source, such as a pathogen (for example, following the infection of a cell by a bacterium or a virus) or from a self-derived protein within a cell, such as a cancerous cell. Examples of sources of self-derived proteins in cancerous cells have been reviewed (Gilboa, E., *Immunity*, 11:263-270, (1999); Rosenberg, S. A., *Immunity*, 10:281-287, (1999)) and include: (i) mutated genes; (ii) aberrantly expressed genes such as an alternative open reading frame or through an intron-exon boundary; (iii) normal genes that

are selectively expressed in only the tumor and the testis; and (iv) normal differentiation genes that are expressed in the tumor and the normal cellular counterpart.

Four different methodologies have typically been used for identifying the peptides that are recognized by CTLs. These are: (i) the genetic method; (2) motif analysis; (3) 5 Serological analysis of REcombinant cDNA expression libraries (SEREX<sup>TM</sup>); and (iv) the immunological and analytical chemistry approach or the Direct Identification of Relevant Epitopes for Clinical Therapeutics (DIRECT<sup>TM</sup>).

The genetic method is an approach in which progressively smaller subsets of cDNA libraries from tumor cells are transfected into cells that express the appropriate MHC 10 molecule but not the tumor-specific epitope. The molecular clones encoding T cell epitopes are identified by their ability to reconstitute tumor specific T cell recognition of transfected cells. The exact T cell epitope is then identified by a combination of molecular subcloning and the use of synthetic peptides based on the predicted amino acid sequence. Such methods, however, are susceptible to inadvertent identification of cross-reacting peptides, and are not 15 capable of identifying important post-translational modifications.

Motif analysis involves scanning a protein for peptides containing known class I MHC binding motifs, followed by synthesis and assay of the predicted peptides for their ability to be recognized by tumor-specific CTL. This approach requires prior knowledge of the protein from which the peptides are derived. This approach is also greatly hampered by 20 the fact that not all of the predicted peptide epitopes are presented on the surface of a cell (Yewdell, J. W. and Bennink, J. R., *Ann.Rev.Immunol.*, 17:51-88, (1999)), thus additional experimentation is required to determine which of the predicted epitopes is useful.

The SEREX<sup>TM</sup> approach relies on using antibodies in the serum of cancer patients to screen cDNA expression libraries for a clone that expresses a protein recognized by the 25 antibody. This methodology presumes that an antibody response will necessarily have developed in the presence of a T cell response, and thus, the identified clone is a good candidate to encode a protein that can be recognized by T cells.

DIRECT<sup>TM</sup> involves a combination of cellular immunology and mass spectrometry. This approach involves the actual identification of endogenous CTL epitopes present on the 30 cell surface by sequencing the naturally occurring peptides associated with class I MHC molecules. In this approach, cells are first lysed in a detergent solution, the peptides associated with the class I MHC molecules are purified, and the peptides are fractionated by high performance liquid chromatography (HPLC). Peptide sequencing is readily performed by tandem mass spectrometry (Henderson, R. A. et al., *Proc.Natl.Acad.Sci.U.S.A.*, 90:10275-

10279, (1993); Hogan, K. T. et al., *Cancer Res.*, 58:5144-5150, (1998); Hunt, D. F. et al., *Science*, 255:1261-1263, (1992); Slingluff, C. L. Jr et al., *J.Immunol.*, 150:2955-2963, (1993)).

Immunization with cancer-derived, class I MHC molecule-associated peptides, or with a parent, or original protein or precursor polypeptide that contains the peptide, or with a gene that encodes a polypeptide or protein containing the peptide, are forms of immunotherapy that can be employed in the treatment of cancer. These forms of immunotherapy require that immunogens be identified so that they can be formulated into an appropriate vaccine. Although a variety of cancer-derived antigens have been identified (Rosenberg, S. A., *Immunity*, 10:281-287, (1999)), not all of these are appropriate for broad-based immunotherapy because the expression of some peptides is limited to the tumor derived from a specific patient. Furthermore, the number of class I MHC molecules from which tumor-derived peptides have been discovered is largely restricted to HLA-A2. Thus, it would be useful to identify additional HLA-A2-restricted peptides. Additionally, it would be useful to identify peptides that complex with class I MHC molecules other than HLA-A2. Such peptides would be particularly useful in the treatment of cancer patients who do not express the HLA-A2 molecule for example HLA-A1/A11 antigens, HLA-A1 supertypes, HLA-A2 supertypes and HLA-A11 supertypes. Identification of and immunization with a cancer-derived parent or original protein or with a gene that encodes the parent protein is significant because the protein can be administered to patients of any HLA type, because proteins that pass through the MHC pathway are processed in vivo to the correct HLA type-specific epitopes.

It is also particularly useful to identify antigenic peptides that are derived from different parent proteins, even if the derived peptides associate with the same class I MHC molecule. Because an active immune response can result in the outgrowth of tumor cells that have lost the expression of a particular precursor protein for a given antigenic peptide, it is advantageous to stimulate an immune response against peptides derived from more than one protein, as the chances of the tumor cell losing the expression of two or more proteins is the multiple of the chances of losing each of the individual proteins.

#### Summary of the Invention

The present invention relates to Immunogens comprising polypeptides with amino acid sequences comprising epitopic sequences selected from the sequences of SEQ ID NO: 1-258 and which immunogens facilitate a cytotoxic T lymphocyte (CTL)-mediated immune response against cancers, especially breast or ovarian cancer. The present invention also

relates to nucleic acid molecules that encode for the polypeptides and/or the full length proteins, their isoforms and splice variants from which the polypeptides are derived, of such immunogens, and which can also be used to facilitate an immune response against cancer.

The present invention provides compositions comprising the immunogen described herein, and polynucleotides that direct the synthesis of such polypeptides, whereby the oligopeptides and polypeptides of such immunogens are capable of inducing a CTL response against cells expressing a protein comprising an epitopic sequence of at least one of SEQ ID NO: 1-258. The cells are usually cancer cells, preferably carcinoma cells, most preferably breast carcinomas or ovarian carcinomas expressing such proteins.

The present invention further relates to polynucleotides comprising the gene coding for a polypeptide of the immunogens disclosed herein. The present invention also provides methods that comprise contacting a lymphocyte, especially a CTL, with an immunogen or its isoforms or splice variants of the invention under conditions that induce a CTL response against a tumor cell, and more specifically against a breast or ovarian tumor cell. The methods may involve contacting the CTL with the immunogenic peptide in vivo, in which case the peptides, polypeptides, and polynucleotides of the invention are used as vaccines, and will be delivered as a pharmaceutical composition comprising a pharmaceutically acceptable carrier or delivery system and the immunogen, typically along with an adjuvant or one or more cytokines.

Alternatively, the immunogens of the present invention can be used to induce a CTL response in vitro. The generated CTL can then be introduced into a patient with cancer, more specifically breast carcinoma, ovarian carcinoma, colorectal carcinoma, lung carcinoma, or prostate carcinoma. Alternatively, the ability to generate CTL in vitro could serve as a diagnostic for cancer generally, including breast carcinoma, ovarian carcinoma, colorectal carcinoma, lung carcinoma, or prostate carcinoma.

#### **Definitions**

As used herein and except as noted otherwise, all terms are defined as given below. The term "peptide" is used herein to designate a series of amino acid residues, connected one to the other typically by peptide bonds between the alpha-amino and carbonyl groups of the adjacent amino acids. The peptides are typically 9 amino acids in length, but can be as short as 8 amino acids in length, and as long as 14 amino acids in length.

The term "oligopeptide" is used herein to designate a series of amino acid residues, connected one to the other typically by peptide bonds between the alpha-amino and carbonyl groups of the adjacent amino acids. The length of the oligopeptide is not critical to the

invention as long as the correct epitope or epitopes are maintained therein. The oligopeptides are typically 30 to about 40 amino acid residues in length, and greater than about 14 amino acids in length.

The term "polypeptide" designates a series of amino acid residues, connected one to the other typically by peptide bonds between the alpha-amino and carbonyl groups of the adjacent amino acids. The length of the polypeptide is not critical to the invention as long as the correct epitopes are maintained. In contrast to the terms peptide or oligopeptide, the term polypeptide is meant to refer to protein molecules of longer than about 40 residues in length.

A peptide, oligopeptide, polypeptide, protein, or polynucleotide coding for such a molecule is "immunogenic" (and thus an "immunogen" within the present invention) if it is capable of inducing an immune response. In the case of the present invention, immunogenicity is more specifically defined as the ability to induce a CTL-mediated response. Thus, an "immunogen" would be a molecule that is capable of inducing an immune response, and in the case of the present invention, a molecule capable of inducing a CTL response. An immunogen may have one or more isoforms or splice variants that have equivalent biological and immunological activity, and are thus also considered for the purposes of this invention to be immunogenic equivalents of the original, natural polypeptide.

A T cell "epitope" is a short peptide molecule that binds to a class I or II MHC molecule and that is subsequently recognized by a T cell. T cell epitopes that bind to class I MHC molecules are typically 8-14 amino acids in length, and most typically 9 amino acids in length. T cell epitopes that bind to class II MHC molecules are typically 12-20 amino acids in length. In the case of epitopes that bind to class II MHC molecules, the same T cell epitope may share a common core segment, but differ in the length of the carboxy- and amino-terminal flanking sequences due to the fact that ends of the peptide molecule are not buried in the structure of the class II MHC molecule peptide-binding cleft as they are in the class I MHC molecule peptide-binding cleft.

Three different genetic loci encode for class I MHC molecules: HLA-A, HLA-B, and HLA-C. HLA-A1, HLA-A2, and HLA-A11 are examples of different class I MHC molecules that can be expressed from these loci. The present invention also involves peptides that are associated with HLA-A1 supertypes, HLA-A2 supertypes, and HLA-A11 supertypes. A supertype is a group of HLA molecules that present at least one shared epitope. MHC molecule peptides that have been found to bind to one member of the MHC



allele supertype family (A1 for example) are thought to be likely to bind to other members of the same supertype family (A32 for example; see Table I, below).

Table I. HLA Supertypes, Motifs and Genotypes

Super type	Motif	Genotypes				
A1	x [ TI (SVLM) ] xxxxxxx [WFY]	A*0101, A*0102, A*2501, A*2601, A*2604, A*3201, A*3601, A*4301, A*8001				
A2	x [LIVMATQ]	A*0201, A*0202,		A*0203		
	xxxxxxx [LIVMAT]	A*0206, A*0207,		A*6802, A*6901		
A3	x [AILMVST] xxxxxxx[RK]	A*0301,	A*1101,	A*3101,	A*3301, A*6801	
A24	x [YF (WIVLMT) ]	A*2301,	A*2402,	A*2403,	A*2404, A*3001,	
	xxxxxxx [EI (YWLM) I]	A*3002,	A*3003			
B7	x [P] xxxxxx [ALIMVFWY]	B*0702,	B*0703,	B*0704,	B*0705, B*1508, B*3501,	
		B*3502,	B*3503,	B*51, B*5301, B*5401, B*5501,		
		B*5502,	B*5601,	B*5602, B*6701,	B*7801	
		B*1401,	B*1402,	B*1503, B*1509,	B*1510,	B*1518,
B27	x [RKH] xxxxxx [FLY (WMI) ]	B*2701,	B*2702,	B*2703, B*2704	B*2705,	B*2706,
		B*2707,	B*2708,	B*3801, B*3802,	B*3901,	B*3902,
		B*3903,	B*3904,	B*4801, B*4802,	B*7301	
				B*4001, B*4006, B*4101	B*4402,	
B44	x [E (D) ] xxxxxx [FWYLIMVA]	B*4403,	B*4501,	B*4901, B*5001		
B58	x [AST] xxxxxx [FWY(LIV)]	B*1516,	B*1517,	B*5701, B*5702,	B*58	
B62	x [QL (IVMP) ] xxxxxxx [FWY (MIV) ]	B*1301,	B*1302,	B*1501, B*1502,	B*1506,	B*1512,
		B*1513,	B*1514,	B*1519, B*1521	B*4601,	B*52

5

As used herein, reference to a DNA sequence includes both single stranded and double stranded DNA. Thus, the specific sequence, unless the context indicates otherwise,

refers to the single strand DNA of such sequence, the duplex of such sequence with its complement (double stranded DNA) and the complement of such sequence.

The term "coding region" refers to that portion of a gene that either naturally or normally codes for the expression product of that gene in its natural genomic environment, i.e., the region coding in vivo for the native expression product of the gene. The coding region can be from a normal, mutated or altered gene, or can even be from a DNA sequence, or gene, wholly synthesized in the laboratory using methods well known to those of skill in the art of DNA synthesis.

The term "nucleotide sequence" refers to a heteropolymer of deoxyribonucleotides. The nucleotide sequence encoding for a particular peptide, oligopeptide, or polypeptide may be naturally occurring or they may be synthetically constructed. Generally, DNA segments encoding the peptides, polypeptides, and proteins of this invention are assembled from cDNA fragments and short oligonucleotide linkers, or from a series of oligonucleotides, to provide a synthetic gene which is capable of being expressed in a recombinant transcriptional unit comprising regulatory elements derived from a microbial or viral operon.

The term "expression product" means that polypeptide or protein that is the natural translation product of the gene and any nucleic acid sequence coding equivalents resulting from genetic code degeneracy and thus coding for the same amino acid(s).

The term "fragment," when referring to a coding sequence, means a portion of DNA comprising less than the complete coding region whose expression product retains essentially the same biological or immunological function or activity as the expression product of the complete coding region.

The term "DNA segment" refers to a DNA polymer, in the form of a separate fragment or as a component of a larger DNA construct, that has been derived from DNA isolated at least once in substantially pure form, i.e., free of contaminating endogenous materials and in a quantity or concentration enabling identification, manipulation, and recovery of the segment and its component nucleotide sequences by standard biochemical methods, for example, by using a cloning vector. Such segments are provided in the form of an open reading frame uninterrupted by internal nontranslated sequences, or introns, which are typically present in eukaryotic genes. Sequences of non-translated DNA may be present downstream from the open reading frame, where the same do not interfere with manipulation or expression of the coding regions. The term "primer" means a short nucleic acid sequence that is paired with one strand of DNA and provides a free 3'OH end at which a DNA polymerase starts synthesis of a deoxyribonucleotide chain.

The term "promoter" means a region of DNA involved in binding of RNA polymerase to initiate transcription.

The term "open reading frame (ORF)" means a series of triplets coding for amino acids without any termination codons and is a sequence (potentially) translatable into protein.

5 The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such  
10 polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

The polynucleotides, and recombinant or immunogenic polypeptides, disclosed in accordance with the present invention may also be in "purified" form. The term "purified" does not require absolute purity; rather, it is intended as a relative definition, and can include  
15 preparations that are highly purified or preparations that are only partially purified, as those terms are understood by those of skill in the relevant art. For example, individual clones isolated from a cDNA library have been conventionally purified to electrophoretic homogeneity. Purification of starting material or natural material to at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of  
20 magnitude is expressly contemplated. Furthermore, the claimed polypeptide which has a purity of preferably 0.001%, or at least 0.01% or 0.1%; and even desirably 1% by weight or greater is expressly contemplated.

The nucleic acids and polypeptide expression products disclosed according to the present invention, as well as expression vectors containing such nucleic acids and/or such  
25 polypeptides, may be in "enriched form." As used herein, the term "enriched" means that the concentration of the material is at least about 2, 5, 10, 100, or 1000 times its natural concentration (for example), advantageously 0.01%, by weight, preferably at least about 0.1% by weight. Enriched preparations of about 0.5%, 1%, 5%, 10%, and 20% by weight are also contemplated. The sequences, constructs, vectors, clones, and other materials comprising  
30 the present invention can advantageously be in enriched or isolated form.

The term "active fragment" means a fragment that generates an immune response (i.e., has immunogenic activity) when administered, alone or optionally with a suitable adjuvant, to an animal, such as a mammal, for example, a human, and also including a rabbit or a mouse, such immune response taking the form of stimulating a CTL response within the recipient,

such as a human. Alternatively, the "active fragment" may also be used to induce a CTL response in vitro.

As used herein, the terms "portion," "segment," and "fragment," when used in relation to polypeptides, refer to a continuous sequence of residues, such as amino acid residues, which sequence forms a subset of a larger sequence. For example, if a polypeptide were subjected to treatment with any of the common endopeptidases, such as trypsin or chymotrypsin, the oligopeptides resulting from such treatment would represent portions, segments or fragments of the starting polypeptide. This means that any such fragment will necessarily contain as part of its amino acid sequence a segment, fragment or portion, that is substantially identical, if not exactly identical, to a sequence of SEQ ID NO: 259-486, which correspond to the naturally occurring original or "parent" proteins of the peptides of SEQ ID NO: 1-258. When used in relation to polynucleotides, such terms refer to the products produced by treatment of said polynucleotides with endonucleases.

In accordance with the present invention, the term "percent identity" or "percent identical," when referring to a sequence, means that a sequence is compared to a claimed or described sequence after alignment of the sequence to be compared (the "Compared Sequence") with the described or claimed sequence (the "Reference Sequence"). The Percent Identity is then determined according to the following formula:

$$\text{Percent Identity} = 100 [1 - (C/R)]$$

wherein C is the number of differences between the Reference Sequence and the Compared Sequence over the length of alignment between the Reference Sequence and the Compared Sequence wherein (i) each base or amino acid in the Reference Sequence that does not have a corresponding aligned base or amino acid in the Compared Sequence and (ii) each gap in the Reference Sequence and (iii) each aligned base or amino acid in the Reference Sequence that is different from an aligned base or amino acid in the Compared Sequence, constitutes a difference; and R is the number of bases or amino acids in the Reference Sequence over the length of the alignment with the Compared Sequence with any gap created in the Reference Sequence also being counted as a base or amino acid.

If an alignment exists between the Compared Sequence and the Reference Sequence for which the percent identity as calculated above is about equal to or greater than a specified minimum Percent Identity then the Compared Sequence has the specified minimum percent identity to the Reference Sequence even though alignments may exist in which the herein above calculated Percent Identity is less than the specified Percent Identity.

Detailed Description of the Invention

The present invention relates generally to immunogens and immunogenic compositions, and methods of use thereof, for the prevention, treatment, and diagnosis of cancer, especially carcinomas, including breast or ovarian carcinomas. Disclosed according  
5 to the invention are immunogens comprising proteins or polypeptides whose amino acid sequences comprises one or more epitopic oligopeptides with sequences selected from the group SEQ ID NO: 1-258. In addition, the invention further relates to polynucleotides that can be used to stimulate a CTL response against cancer, and more specifically carcinoma, especially breast or ovarian carcinomas.

10 In accordance with the present invention there are disclosed specific oligopeptide sequences with amino acid sequences shown in SEQ ID NO: 1-258 which represent epitopic peptides (i.e. immunogenic oligopeptide sequences) of at least about 8 amino acids in length, preferably about 9 amino acids in length (i.e., nonapeptides), and no longer than about 14  
15 amino acids in length and present as part of a larger structure, such as a polypeptide or full length protein.

While the use of specific peptides is restricted to use in patients having certain HLA types or HLA supertypes, there is no such restriction on the use of the parent protein as an immunogen. When the parent protein or immunogen is presented to the antigen processing  
20 pathway, it will be appropriately fragmented, processed and presented in the context of HLA type(s) present in the patient.

The polypeptides forming the immunogens of the present invention have amino acid sequences that comprise at least one stretch, possibly two, three, four, or more stretches of about 8 to 10 or up to 14 residues in length and which stretches differ in amino acid sequence  
25 from the sequences of SEQ ID NO: 1-258 by no more than about 1 amino acid residue, preferably a conservative amino acid residue, especially amino acids of the same general chemical character, such as where they are hydrophobic amino acids.

Said polypeptides can be of any desired length so long as they have immunogenic activity in that they are able, under a given set of desirable conditions, to elicit in vitro or in  
30 vivo the activation of cytotoxic T lymphocytes (CTLs) (i.e., a CTL response) against a presentation of a cancer specific protein, especially a carcinoma or sarcoma specific protein where said proteins are presented in vitro or in vivo by an antigen presenting cell (APC). The proteins and polypeptides forming the immunogens of the present invention can be naturally

occurring or may be synthesized chemically. According to the present invention the polypeptides may comprise at least one of SEQ ID NO: 259 to 486.

The present invention is also directed to an isolated polypeptide, especially one having immunogenic activity, the sequence of which comprises within it one or more stretches comprising any 2 or more of the sequences of SEQ ID NO: 1-258 and in any relative quantities and wherein said sequences may differ by one amino acid residues from the sequences of SEQ ID NO: 1-258 in any given stretch of 8 to 10, or up to 14 amino acid residues. Thus, within the present invention, by way of a non-limiting example only, such polypeptide may contain as part of its amino acid sequence, nonapeptide fragments having up to 8 amino acids identical to a sequence of SEQ ID NO: 1,2,7,8 such that the polypeptide comprises, in a specific embodiment, 2 segments with at least 8 residues identical to SEQ ID NO: 1 and SEQ ID NO: 2 and one segment with at least 8 residues identical to SEQ ID NO: 7. In other embodiments, other combinations and permutations of the epitopic sequences disclosed herein may be part of an immunogen of the present invention or of such a polypeptide so long as any such polypeptide comprises at least 2 such epitopes, whether such epitopes are different or the same. Thus, in a specific embodiment, a polypeptide of the present invention may comprise 2 copies of the sequence of SEQ ID NO: 2 at some point or points within its length. Of course, any combinations and permutations of the epitopes disclosed herein, as long as they are present at least two in number in such polypeptides, are expressly contemplated.

All of the epitopic peptides of SEQ ID NO: 1-258 are derived from proteins expressed by cancer cells and sequences and were identified through the method of Automated High Through-put Sequencing (HTPS). Accordingly, SEQ ID NO: 259-486 are polypeptides that comprise at least one of SEQ ID NO: 1-258.

Oligopeptides as disclosed herein may themselves be prepared by methods well known to those skilled in the art. (Grant, G. A., *Synthetic Peptides: A User's Guide*, 1992, W. H. Freeman and Company, New York; Coligan, J. E. et al, *Current Protocols in Protein Science*, 1999, John Wiley & Sons, Inc., New York).

Besides the sequences of SEQ ID NO:1-258, the proteins and polypeptides forming the immunogens of the present invention may also comprise one or more other immunogenic amino acid stretches known to be associated with cancer, and more specifically with carcinomas including breast carcinoma, ovarian carcinoma, colorectal carcinoma, lung carcinoma, or prostate carcinoma, and which may stimulate a CTL response whereby the

immunogenic peptides associate with HLA-A2, HLA-A1/A11, HLA supertypes, or any class I MHC (i.e., MHC-I) molecule.

The immunogens of the present invention can be in the form of a composition of one or more of the different immunogens and wherein each immunogen is present in any desired relative abundance. Such compositions can be homogeneous or heterogeneous with respect to the individual immunogenic peptide components present therein, having only one or more than one of such peptides.

The oligopeptides and polypeptides useful in practicing the present invention may be derived by fractionation of naturally occurring proteins by methods such as protease treatment, or they may be produced by recombinant or synthetic methodologies that are well known and clear to the skilled artisan (Ausubel, F. M. et al, Current Protocols in Molecular Biology, 1999, John Wiley & Sons, Inc., New York; Coligan, J. E. et al, Current Protocols in Protein Science, 1999, John Wiley & Sons, Inc., New York; Molecular Cloning: A Laboratory Manual, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor). The polypeptide may comprise a recombinant or synthetic polypeptide that comprises at least one of SEQ ID NO:1-258 which sequences may also be present in multiple copies. Thus, oligopeptides and polypeptides of the present invention may have one, two, three, or more such immunogenic peptides within the amino acid sequence of said oligopeptides and polypeptides, and said immunogenic peptides, or epitopes, may be the same or may be different, or may have any number of such sequences wherein some of them are identical to each other in amino acid sequence while others within the same polypeptide sequence are different from each other and said epitopic sequences may occur in any order within said immunogenic polypeptide sequence. The location of such sequences within the sequence of a polypeptide forming an immunogen of the invention may affect relative immunogenic activity. In addition, immunogens of the present invention may comprise more than one protein comprising the amino acid sequences disclosed herein. Such polypeptides may be part of a single composition or may themselves be covalently or non-covalently linked to each other.

The immunogenic peptides disclosed herein may also be linked directly to, or through a spacer or linker to: an immunogenic carrier such as serum albumin, tetanus toxoid, keyhole limpet hemocyanin, dextran, or a recombinant virus particle; an immunogenic peptide known to stimulate a T helper cell type immune response; a cytokine such as interferon gamma or GM-CSF; a targeting agent such as an antibody or receptor ligand; a stabilizing agent such as a lipid; or a conjugate of a plurality of epitopes to a branched lysine core structure, such as

the so-called "multiple antigenic peptide" described in (Posnelt, D. N. et al., J.Biol.Chem., 263:1719-1725, (1988)); a compound such as polyethylene glycol to increase the half life of the peptide; or additional amino acids such as a leader or secretory sequence, or a sequence employed for the purification of the mature sequence. Spacers and linkers typically comprise

5 relatively small, neutral molecules, such as amino acids and which are substantially uncharged under physiological conditions. Such spacers are typically selected from the group of nonpolar or neutral polar amino acids, such as glycine, alanine, serine and other similar amino acids. Such optional spacers or linkers need not comprise the same residues and thus may be either homo- or hetero-oligomers. When present, such linkers will commonly be of

10 length at least one or two, commonly 3, 4, 5, 6, and possibly as much as 10 or even up to 20 residues (in the case of amino acids). In addition, such linkers need not be composed of amino acids but any oligomeric structures will do as well so long as they provide the correct spacing so as to optimize the desired level of immunogenic activity of the immunogens of the present invention. The immunogen may therefore take any form that is capable of eliciting a

15 CTL response.

In addition, the immunogenic peptides of the present invention may be part of an immunogenic structure via attachments other than conventional peptide bonds. Thus, any manner of attaching the peptides of the invention to an immunogen of the invention, such as an immunogenic polypeptide as disclosed herein, could provide an immunogenic structure as

20 claimed herein. Thus, immunogens, such as proteins, oligopeptides and polypeptides of the invention, are structures that contain the peptides disclosed according to the present invention but such immunogenic peptides may not necessarily be attached thereto by the conventional means of using ordinary peptide bounds. The immunogens of the present invention simply contain such peptides as part of their makeup, but how such peptides are to be combined to

25 form the final immunogen is left to the talent and imagination of the user and is in no way restricted or limited by the disclosure contained herein.

The peptides that are naturally processed and bound to a class I MHC molecule, and which are recognized by a tumor-specific CTL, need not be the optimal peptides for stimulating a CTL response. See, for example, (Parkhurst, M. R. et al., J.Immunol., 157:2539-2548, (1996); Rosenberg, S. A. et al., Nat.Med., 4:321-327, (1998)). Thus, there

30 can be utility in modifying a peptide, such that it more readily induces a CTL response. Generally, peptides may be modified at two types of positions. The peptides may be modified at amino acid residues that are predicted to interact with the class I MHC molecule, in which case the goal is to create a peptide that has a higher affinity for the class I MHC molecule



than does the original peptide. The peptides can also be modified at amino acid residues that are predicted to interact with the T cell receptor on the CTL, in which case the goal is to create a peptide that has a higher affinity for the T cell receptor than does the original peptide. Both of these types of modifications can result in a variant peptide that is related to an original peptide, but which is better able to induce a CTL response than is the original peptide. As used herein, the term "original peptide" means an oligopeptide with the amino acid sequence selected from SEQ ID NO: 1-258.

The original peptides disclosed herein can be modified by the substitution of one or more residues at different, possibly selective, sites within the peptide chain. Such substitutions may be of a conservative nature, for example, where one amino acid is replaced by an amino acid of similar structure and characteristics, such as where a hydrophobic amino acid is replaced by another hydrophobic amino acid. Even more conservative would be replacement of amino acids of the same or similar size and chemical nature, such as where leucine is replaced by isoleucine. In studies of sequence variations in families of naturally occurring homologous proteins, certain amino acid substitutions are more often tolerated than others, and these often show correlation with similarities in size, charge, polarity, and hydrophobicity between the original amino acid and its replacement, and such is the basis for defining "conservative substitutions."

Conservative substitutions are herein defined as exchanges within one of the following five groups: Group 1--small aliphatic, nonpolar or slightly polar residues (Ala, Ser, Thr, Pro, Gly); Group 2--polar, negatively charged residues and their amides (Asp, Asn, Glu, Gln); Group 3--polar, positively charged residues (His, Arg, Lys); Group 4--large, aliphatic, nonpolar residues (Met, Leu, Ile, Val, Cys); and Group 4--large, aromatic residues (Phe, Tyr, Trp).

Less conservative substitutions might involve the replacement of one amino acid by another that has similar characteristics but is somewhat different in size, such as replacement of an alanine by an isoleucine residue. Highly nonconservative replacements might involve substituting an acidic amino acid for one that is polar, or even for one that is basic in character. Such radical substitutions cannot, however, be dismissed as potentially ineffective since chemical effects are not totally predictable and radical substitutions might well give rise to serendipitous effects not otherwise predictable from simple chemical principles.

Of course, such substitutions may involve structures other than the common L-amino acids. Thus, D-amino acids might be substituted for the L-amino acids commonly found in the antigenic peptides of the invention and yet still be encompassed by the disclosure herein.

In addition, amino acids possessing non-standard R groups (i.e., R groups other than those found in the common 20 amino acids of natural proteins) may also be used for substitution purposes to produce immunogens and immunogenic polypeptides according to the present invention.

5 If substitutions at more than one position are found to result in a peptide with substantially equivalent or greater antigenic activity as defined below, then combinations of those substitutions will be tested to determine if the combined substitutions result in additive or synergistic effects on the antigenicity of the peptide. At most, no more than 4 positions within the peptide would simultaneously be substituted.

10 Based on cytotoxicity assays, an epitope is considered substantially identical to the reference peptide if it has at least 10% of the antigenic activity of the reference peptide as defined by the ability of the substituted peptide to reconstitute the epitope recognized by a CTL in comparison to the reference peptide. Thus, when comparing the lytic activity in the linear portion of the effector:target curves with equimolar concentrations of the reference and substituted peptides, the observed percent specific killing of the target cells incubated with  
15 the substituted peptide should be equal to that of the reference peptide at an effector:target ratio that is no greater than 10-fold above the reference peptide effector:target ratio at which the comparison is being made.

Preferably, when the CTLs specific for a peptide of SEQ ID NO:1-258 are tested  
20 against the substituted peptides, the peptide concentration at which the substituted peptides achieve half the maximal increase in lysis relative to background is no more than about 1 mM, preferably no more than about 1  $\mu$ M, more preferably no more than about 1 nM, and still more preferably no more than about 100 pM, and most preferably no more than about 10 pM. It is also preferred that the substituted peptide be recognized by CTLs from more than  
25 one individual, at least two, and more preferably three individuals.

Thus, the epitopes of the present invention may be identical to naturally occurring tumor-associated or tumor-specific epitopes or may include epitopes that differ by no more than 4 residues from the reference peptide, as long as they have substantially identical antigenic activity.

30 It should be appreciated that an immunogen may consist only of a peptide of SEQ ID NO:1-258, or comprise a peptide of SEQ ID NO:1-258, or comprise a plurality of peptides selected from SEQ ID NO:1-258, or comprise a polypeptide that itself comprises one or more of the epitopic peptides of SEQ ID NO: 1-258.

The immunogenic peptides and polypeptides of the invention can be prepared synthetically, by recombinant DNA technology, or they can be isolated from natural sources such as tumor cells expressing the original protein product.

The polypeptides and oligopeptides disclosed herein can be synthesized in solution or on a solid support in accordance with conventional techniques. Various automated peptide synthesizers are commercially available and can be used in accordance with known protocols. See, for example, (Grant, G. A., *Synthetic Peptides: A User's Guide*, 1992, W. H. Freeman and Company, New York; Coligan, J. E. et al, *Current Protocols in Protein Science*, 1999, John Wiley & Sons, Inc., New York). Fragments of polypeptides of the invention can also be synthesized as intermediates in the synthesis of a larger polypeptide.

Recombinant DNA technology may be employed wherein a nucleotide sequence that encodes an immunogenic peptide or polypeptide of interest is inserted into an expression vector, transformed or transfected into an appropriate host cell, and cultivated under conditions suitable for expression. These procedures are well known in the art to the skilled artisan, as described in (Coligan, J. E. et al, *Current Protocols in Immunology*, 1999, John Wiley & Sons, Inc., New York; Ausubel, F. M. et al, *Current Protocols in Molecular Biology*, 1999, John Wiley & Sons, Inc., New York; *Molecular Cloning: A Laboratory Manual*, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor). Thus, recombinantly produced peptides or polypeptides can be used as the immunogens of the invention.

The coding sequences for peptides of the length contemplated herein can be synthesized on commercially available automated DNA synthesizers using protocols that are well known in the art. See for example, (Grant, G. A., *Synthetic Peptides: A User's Guide*, 1992, W. H. Freeman and Company, New York; Coligan, J. E. et al, *Current Protocols in Protein Science*, 1999, John Wiley & Sons, Inc., New York). The coding sequences can also be modified such that a peptide or polypeptide will be produced that incorporates a desired amino acid substitution. The coding sequence can be provided with appropriate linkers, be ligated into suitable expression vectors that are commonly available in the art, and the resulting DNA or RNA molecule can be transformed or transfected into suitable hosts to produce the desired fusion protein. A number of such vectors and suitable host systems are available, and their selection is left to the skilled artisan. For expression of the fusion proteins, the coding sequence will be provided with operably linked start and stop codons, promoter and terminator regions, and a replication system to provide an expression vector for expression in the desired host cell. For example, promoter sequences compatible with bacterial hosts are provided in plasmids containing convenient restriction sites for insertion of

the desired coding sequence. The resulting expression vectors are transformed into suitable bacterial hosts. Yeast, insect, and mammalian host cells may also be used, employing suitable vectors and control sequences.

Host cells are genetically engineered (transduced or transformed or transfected) with the vectors of this invention which may be, for example, a cloning vector or an expression vector. The vector may be, for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the genes of the present invention. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

More particularly, the present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available.

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Ausubel, F. M. et al, Current Protocols in Molecular Biology, 1999, John Wiley & Sons, Inc., New York; Molecular Cloning: A Laboratory Manual, 1989, Cold Spring Harbor Laboratory Press, Cold Spring Harbor). Such cells can routinely be utilized for assaying CTL activity by having said genetically engineered, or recombinant, host cells express the immunogenic peptides of the present invention.

Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell, 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites,

polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking non-transcribed sequences. DNA sequences derived from the SV40 splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

5       The polypeptide can be recovered and purified from recombinant cell cultures by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps can be used, as necessary, in completing  
10       configuration of the mature protein. High performance liquid chromatography (HPLC) can be employed for final purification steps.

      The immunogenic peptides of the present invention may be used to elicit CTLs ex vivo from either healthy individuals or from cancer patients, such as breast carcinoma, colorectal carcinoma, lung carcinoma, ovarian carcinoma, or prostate carcinoma. Such  
15       responses are induced by incubating in tissue culture the individual's CTL precursor lymphocytes together with a source of antigen presenting cells and the appropriate immunogenic peptide. Examples of suitable antigen presenting cells include dendritic cells, macrophages, and activated B cells. Typically, the peptide at concentrations between 10 and 40 µg/ml, would be pre-incubated with the antigen presenting cells for periods ranging from 1  
20       to 18 hrs.  $\beta_2$ -microglobulin (4 µg/ml) can be added during this time period to enhance binding. The antigen presenting cells may also be held at room temperature during the incubation period (Ljunggren, H.-G. et al., *Nature*, 346:476-480, (1990)) or pretreated with acid (Zeh, H. J., III et al., *Hum. Immunol.*, 39:79-86, (1994)) to promote the generation of denatured class I MHC molecules that can then bind the peptide. The precursor CTLs  
25       (responders) are then added to the antigen presenting cells to which the immunogenic peptide has bound (stimulators) at responder to stimulator ratios of between 5:1 and 50:1, and most typically between 10:1 and 20:1. The co-cultivation of the cells is carried out at 37° C. in RPMI 1640, 10% fetal bovine serum, 2 mM L-glutamine, and IL-2 (5-20 Units/ml). Other cytokines, such as IL-1, IL-7, and IL-12 may also be added to the culture. Fresh IL-2-  
30       containing media is added to the cultures every 2-4 days, typically by removing one-half the old media and replenishing it with an equal volume of fresh media. After 7-10 days, and every 7-10 days thereafter, the CTL are re-stimulated with antigen presenting cells to which immunogenic peptide has been bound as described above. Fresh IL-2-containing media is added to the cells throughout their culture as described above. Three to four rounds of

stimulation, and sometimes as many five to eight rounds of stimulation, are required to generate a CTL response that can then be measured in vitro. The above described protocol is illustrative only and should not be considered limiting. Many in vitro CTL stimulation protocols have been described and the choice of which one to use is well within the knowledge of the skilled artisan. The peptide-specific CTL can be further expanded to large numbers by treatment with anti-CD3 antibody. For example, see (Riddell, S. R. and Greenberg, P. D., *J.Immunol.Methods*, 128:189-201, (1990); Walter, E. A. et al., *N.Engl.J.Med.*, 333:1038-1044, (1995)).

Antigen presenting cells that are to be used to stimulate a CTL response are typically incubated with peptide of an optimal length, for example a nonapeptide, that allows for direct binding of the peptide to the class I MHC molecule without additional processing. Larger oligopeptides and polypeptides are generally ineffective in binding to class I MHC molecules as they are not efficiently processed into an appropriately sized peptide in the extracellular milieu. A variety of approaches are known in the art, however, that allow oligopeptides and polypeptides to be exogenously acquired by a cell, which then allows for their subsequent processing and presentation by a class I MHC molecule. Representative, but non-limiting examples of such approaches include electroporation of the molecules into the cell (Harding, C. H. III, *Eur.J.Immunol.*, 22:1865-1869, (1992)), encapsulation of the molecules in liposomes that are fused to the cells of interest (Reddy, R. et al., *J.Immunol.Methods*, 141:157-163, (1991)), or osmotic shock in which the molecules are taken up via pinocytosis (Moore, M. W. et al., *Cell*, 54:777-785, (1988)). Thus, oligopeptides and polypeptides that comprise one or more of the peptides of the invention can be provided to antigen presenting cells in such a fashion that they are delivered to the cytoplasm of the cell, and are subsequently processed to allow presentation of the peptides.

Antigen presenting cells suitable for stimulating an in vitro CTL response that is specific for one or more of the peptides of the invention can also be prepared by introducing polynucleotide vectors encoding the sequences into the cells. These polynucleotides can be designed such that they express only a single peptide of the invention, multiple peptides of the invention, or even a plurality of peptides of the invention. A variety of approaches are known in the art that allow polynucleotides to be introduced and expressed in a cell, thus providing one or more peptides of the invention to the class I MHC molecule binding pathway. Representative, but non-limiting examples of such approaches include the introduction of plasmid DNA through particle-mediated gene transfer or electroporation (Tuting, T. et al., *J.Immunol.*, 160:1139-1147, (1998)), or the transduction of cells with an

adenovirus expressing the polynucleotide of interest (Perez-Diez, A. et al., Cancer Res., 58:5305-5309, (1998)). Thus, oligonucleotides that code for one or more of the peptides of the invention can be provided to antigen presenting cells in such a fashion that the peptides associate with class I MHC molecules and are presented on the surface of the antigen presenting cell, and consequently are available to stimulate a CTL response.

By preparing the stimulator cells used to generate an in vitro CTL response in different ways, it is possible to control the peptide specificity of CTL response. For example, the CTLs generated with a particular peptide will necessarily be specific for that peptide. Likewise, CTLs that are generated with a polypeptide or polynucleotide expressing or coding for particular peptides will be limited to specificities that recognize those peptides. More broadly, stimulator cells, and more specifically dendritic cells, can be incubated in the presence of the whole parent protein. As a further alternative, stimulator cells, and more specifically dendritic cells, can be transduced or transfected with RNA or DNA comprising the polynucleotide sequence encoding the protein. Under these alternative conditions, peptide epitopes that are naturally cleaved out of the protein, and which are generated in addition to peptide epitopes of SEQ ID NO:1-258 can associate with an appropriate class I MHC molecule, which may or may not include HLA-A1, -A2, -A3. The selection of antigen presenting cells and the type of antigen with which to stimulate the CTL, is left to the ordinary skilled artisan.

In certain embodiments, the methods of the present invention include a method for inducing a CTL response in vitro that is specific for a tumor cell expressing a molecule from A1, A2, or A3 supertypes (A11 is a member of the A3 supertype), whereby the method comprises contacting a CTL precursor lymphocyte with an antigen presenting cell that has bound an immunogen comprising one or more of the peptides disclosed according to the invention.

In specific embodiments, the methods of the present invention include a method for inducing a CTL response in vitro that is specific for a tumor cell expressing a molecule from A1, A2, or A3 supertypes, whereby the method comprises contacting a CTL precursor lymphocyte with an antigen presenting cell that has exogenously acquired an immunogenic oligopeptide or polypeptide that comprises one or more of the peptides disclosed according to the invention.

A yet additional embodiment of the present invention is directed to a process for inducing a CTL response in vitro that is specific for a tumor cell expressing a molecule from

A1, A2, or A3 supertypes, comprising contacting a CTL precursor lymphocyte with an antigen presenting cell that is expressing a polynucleotide coding for a polypeptide of the invention and wherein said polynucleotide is operably linked to a promoter.

A variety of techniques exist for assaying the activity of CTL. These techniques include the labeling of target cells with radionuclides such as  $\text{Na}_2^{51}\text{CrO}_4$  or  $^3\text{H}$ -thymidine, and measuring the release or retention of the radionuclides from the target cells as an index of cell death. Such assays are well-known in the art and their selection is left to the skilled artisan. Alternatively, CTL are known to release a variety of cytokines when they are stimulated by an appropriate target cell, such as a tumor cell expressing the relevant class I MHC molecule and the corresponding peptide. Non-limiting examples of such cytokines include IFN- $\gamma$ , TNF- $\alpha$ , and GM-CSF. Assays for these cytokines are well known in the art, and their selection is left to the skilled artisan. Methodology for measuring both target cell death and cytokine release as a measure of CTL reactivity are given in Coligan, J. E. et al. (Current Protocols in Immunology, 1999, John Wiley & Sons, Inc., New York).

After expansion of the antigen-specific CTLs, the latter are then adoptively transferred back into the patient, where they will destroy their specific target cell. The utility of such adoptive transfer is demonstrated in North, R. J. et al. (Infect.Immun., 67:2010-2012, (1999)) and Riddell, S. R. et al. (Science, 257:238-241, (1992)). In determining the amount of cells to reinfuse, the skilled physician will be guided by the total number of cells available, the activity of the CTL as measured in vitro, and the condition of the patient. Preferably, however, about  $1 \times 10^6$  to about  $1 \times 10^{12}$ , more preferably about  $1 \times 10^8$  to about  $1 \times 10^{11}$ , and even more preferably, about  $1 \times 10^9$  to about  $1 \times 10^{10}$  peptide-specific CTL are infused. Methodology for reinfusing T cells into a patient are well known and exemplified in U.S. Pat. No. 4,844,893 to Honski, et al., and U.S. Pat. No. 4,690,915 to Rosenberg.

The peptide-specific CTL can be purified from the stimulator cells prior to infusion into the patient. For example, monoclonal antibodies directed toward the cell surface protein CD8, present on CTL, can be used in conjunction with a variety of isolation techniques such as antibody panning, flow cytometric sorting, and magnetic bead separation to purify the peptide-specific CTL away from any remaining non-peptide specific lymphocytes or from the stimulator cells. These methods are well known in the art, and their selection is left to the skilled artisan. It should be appreciated that generation of peptide-specific CTL in this manner obviates the need for stimulating the CTL in the presence of tumor. Thus, there is no chance of inadvertently reintroducing tumor cells into the patient.



Thus, one embodiment of the present invention relates to a process for treating a subject with cancer characterized by tumor cells expressing complexes of a molecule from A1, A2, or A3 supertypes, for example, HLA-A1, HLA-A2, or HLA-A11, whereby CTLs produced in vitro according to the present invention are administered in an amount sufficient to destroy the tumor cells through direct lysis or to effect the destruction of the tumor cells indirectly through the elaboration of cytokines.

Another embodiment of the present invention is directed to a process for treating a subject with cancer characterized by tumor cells expressing any class I MHC molecule and an epitope of SEQ ID NO: 1-258, whereby the CTLs are produced in vitro and are specific for the epitope or original protein and are administered in an amount sufficient to destroy the tumor cells through direct lysis or to effect the destruction of the tumor cells indirectly through the elaboration of cytokines.

In the foregoing embodiments the cancer to be treated may include a breast carcinoma, a colorectal carcinoma, an ovarian carcinoma, a lung carcinoma, and prostate carcinoma, but especially breast carcinoma.

The ex vivo generated CTL can be used to identify and isolate the T cell receptor molecules specific for the peptide. The genes encoding the alpha and beta chains of the T cell receptor can be cloned into an expression vector system and transferred and expressed in naive T cells from peripheral blood, T cells from lymph nodes, or T lymphocyte progenitor cells from bone marrow. These T cells, which would then be expressing a peptide-specific T cell receptor, would then have anti-tumor reactivity and could be used in adoptive therapy of cancer, and more specifically cancer, breast carcinoma, colorectal carcinoma, ovarian carcinoma, lung carcinoma, and prostate carcinoma.

In addition to their use for therapeutic or prophylactic purposes, the immunogenic peptides of the present invention are useful as screening and diagnostic agents. Thus, the immunogenic peptides of the present invention, together with modern techniques of gene screening, make it possible to screen patients for the presence of genes encoding such peptides on cells obtained by biopsy of tumors detected in such patients. The results of such screening may help determine the efficacy of proceeding with the regimen of treatment disclosed herein using the immunogens of the present invention.

Alternatively, the immunogenic peptides disclosed herein, as well as functionally similar homologs thereof, may be used to screen a sample for the presence of CTLs that specifically recognize the corresponding epitopes. The lymphocytes to be screened in this assay will normally be obtained from the peripheral blood, but lymphocytes can be obtained

from other sources, including lymph nodes, spleen, tumors, and pleural fluid. The peptides of the present invention may then be used as a diagnostic tool to evaluate the efficacy of the immunotherapeutic treatments disclosed herein. Thus, the in vitro generation of CTL as described above would be used to determine if patients are likely to respond to the peptide in vivo. Similarly, the in vitro generation of CTL could be done with samples of lymphocytes obtained from the patient before and after treatment with the peptides. Successful generation of CTL in vivo should then be recognized by a correspondingly easier ability to generate peptide-specific CTL in vitro from lymphocytes obtained following treatment in comparison to those obtained before treatment.

The oligopeptides of the invention, such as SEQ ID NO: 1-258, can also be used to prepare class I MHC tetramers which can be used in conjunction with flow cytometry to quantitate the frequency of peptide-specific CTL that are present in a sample of lymphocytes from an individual. Specifically, for example, class I MHC molecules comprising peptides of SEQ ID NO: 1-258, would be combined to form tetramers as exemplified in U.S. Pat. No. 5,635,363. Said tetramers would find use in monitoring the frequency of CTLs in the peripheral blood, lymph nodes, or tumor mass of an individual undergoing immunotherapy with the peptides, proteins, or polynucleotides of the invention, and it would be expected that successful immunization would lead to an increase in the frequency of the peptide-specific CTL.

As stated above, a vaccine in accordance with the present invention may include one or more of the hereinabove described polypeptides or active fragments thereof, or a composition, or pool, of immunogenic peptides disclosed herein. When employing more than one polypeptide or active fragment, such as two or more polypeptides and/or active fragments may be used as a physical mixture or as a fusion of two or more polypeptides or active fragments. The fusion fragment or fusion polypeptide may be produced, for example, by recombinant techniques or by the use of appropriate linkers for fusing previously prepared polypeptides or active fragments.

The immunogenic molecules of the invention, including vaccine compositions, may be utilized according to the present invention for purposes of preventing, suppressing or treating diseases causing the expression of the immunogenic peptides disclosed herein, such as where the antigen is being expressed by tumor cells. As used in accordance with the present invention, the term "prevention" relates to a process of prophylaxis in which an animal, especially a mammal, and most especially a human, is exposed to an immunogen of the present invention prior to the induction or onset of the disease process. This could be

done where an individual has a genetic pedigree indicating a predisposition toward occurrence of the disease condition to be prevented. For example, this might be true of an individual whose ancestors show a predisposition toward certain types of cancer. Alternatively, the immunogen could be administered to the general population as is frequently done for infectious diseases. Alternatively, the term "suppression" is often used to describe a condition wherein the disease process has already begun but obvious symptoms of said condition have yet to be realized. Thus, the cells of an individual may have become cancerous but no outside signs of the disease have yet been clinically recognized. In either case, the term prophylaxis can be applied to encompass both prevention and suppression. Conversely, the term "treatment" is often utilized to mean the clinical application of agents to combat an already existing condition whose clinical presentation has already been realized in a patient. This would occur where an individual has already been diagnosed as having a tumor.

It is understood that the suitable dosage of an immunogen of the present invention will depend upon the age, sex, health, and weight of the recipient, the kind of concurrent treatment, if any, the frequency of treatment, and the nature of the effect desired. However, the most preferred dosage can be tailored to the individual subject, as determined by the researcher or clinician. The total dose required for any given treatment will commonly be determined with respect to a standard reference dose as set by a manufacturer, such as is commonly done with vaccines, such dose being administered either in a single treatment or in a series of doses, the success of which will depend on the production of a desired immunological result (i.e., successful production of a CTL-mediated response to the antigen, which response gives rise to the prevention and/or treatment desired). Thus, the overall administration schedule must be considered in determining the success of a course of treatment and not whether a single dose, given in isolation, would or would not produce the desired immunologically therapeutic result or effect.

The therapeutically effective amount of a composition containing one or more of the immunogens of this invention, is an amount sufficient to induce an effective CTL response to cure or arrest disease progression. Thus, this dose will depend, among other things, on the identity of the immunogens used, the nature of the disease condition, the severity of the disease condition, the extent of any need to prevent such a condition where it has not already been detected, the manner of administration dictated by the situation requiring such administration, the weight and state of health of the individual receiving such administration, and the sound judgment of the clinician or researcher. Thus, for purposes of prophylactic or

therapeutic administration, effective amounts would generally lie within the range of from 1.0 µg to about 5,000 µg of peptide for a 70 kg patient, followed by boosting dosages of from about 1.0 µg to about 1,000 µg of peptide pursuant to a boosting regimen over days, weeks or months, depending on the recipient's response and as necessitated by subsequent monitoring of CTL-mediated activity within the bloodstream. Of course, such dosages are to be considered only a general guide and, in a given situation, may greatly exceed such suggested dosage regimens where the clinician believes that the recipient's condition warrants more aggressive administration schedule. The efficacy of administering additional doses, and of increasing or decreasing the interval, may be re-evaluated on a continuing basis, in view of the recipient's immunocompetence (for example, the level of CTL activity with respect to tumor-associated or tumor-specific antigens).

For such purposes, the immunogenic compositions according to the present invention may be used against a disease condition such as cancer by administration to an individual by a variety of routes. The composition may be administered parenterally or orally, and, if parenterally, either systemically or topically. Parenteral routes include subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intranasal, transdermal, or buccal routes. One or more such routes may be employed. Parenteral administration can be, for example, by bolus injection or by gradual perfusion over time.

Generally, vaccines are prepared as injectables, in the form of aqueous solutions or suspensions. Vaccines in an oil base are also well known such as for inhaling. Solid forms that are dissolved or suspended prior to use may also be formulated. Pharmaceutical carriers, diluents and excipients are generally added that are compatible with the active ingredients and acceptable for pharmaceutical use. Examples of such carriers include, but are not limited to, water, saline solutions, dextrose, or glycerol. Combinations of carriers may also be used. These compositions may be sterilized by conventional, well known sterilization techniques including sterile filtration. The resulting solutions may be packaged for use as is, or the aqueous solutions may be lyophilized, the lyophilized preparation being combined with sterile water before administration. Vaccine compositions may further incorporate additional substances to stabilize pH, or to function as adjuvants, wetting agents, or emulsifying agents, which can serve to improve the effectiveness of the vaccine.

The concentration of the CTL stimulatory peptides of the invention in pharmaceutical formulations are subject to wide variation, including anywhere from less than 0.01% by weight to as much as 50% or more. Factors such as volume and viscosity of the resulting composition must also be considered. The solvents, or diluents, used for such compositions

include water, dimethylsulfoxide, PBS (phosphate buffered saline), or saline itself, or other possible carriers or excipients.

The immunogens of the present invention may also be contained in artificially created structures such as liposomes, ISCOMS, slow-releasing particles, and other vehicles which increase the immunogenicity and/or half-life of the peptides or polypeptides in serum. Liposomes include emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. Liposomes for use in the invention are formed from standard vesicle-forming lipids which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally determined by considerations such as liposome size and stability in the blood. A variety of methods are available for preparing liposomes as reviewed, for example, by (Coligan, J. E. et al, Current Protocols in Protein Science, 1999, John Wiley & Sons, Inc., New York) and see also U.S. Pat. Nos. 4,235,871, 4,501,728, 4,837,028, and 5,019,369. Liposomes containing the peptides or polypeptides of the invention can be directed to the site of lymphoid cells where the liposomes then deliver the selected immunogens directly to antigen presenting cells. Targeting can be achieved by incorporating additional molecules such as proteins or polysaccharides into the outer membranes of said structures, thus resulting in the delivery of the structures to particular areas of the body, or to particular cells within a given organ or tissue. Such targeting molecules may a molecule that binds to receptor on antigen presenting cells. For example an antibody that binds to CD80 could be used to direct liposomes to dendritic cells.

The immunogens of the present invention may also be administered as solid compositions. Conventional nontoxic solid carriers including pharmaceutical grades of mannitol, lactose, starch, magnesium, cellulose, glucose, sucrose, sodium saccharin, and the like. Such solid compositions will often be administered orally, whereby a pharmaceutically acceptable nontoxic composition is formed by incorporating the peptides and polypeptides of the invention with any of the carriers listed above. Generally, such compositions will contain 10-95% active ingredient, and more preferably 25-75% active ingredient.

Aerosol administration is also an alternative, requiring only that the immunogens be properly dispersed within the aerosol propellant. Typical percentages of the peptides or polypeptides of the invention are 0.01%-20% by weight, preferably 1% -10%. The use of a surfactant to properly disperse the immunogen may be required. Representative surfactants include the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an

aliphatic polyhydric alcohol or its cyclic anhydride. Mixed esters, such as mixed or natural glycerides may be employed. The surfactant may constitute 0.1-20% by weight of the composition, preferably 0.25-5%. Typical propellants for such administration may include esters and similar chemicals but are by no means limited to these. A carrier, such as lecithin  
5 for intranasal delivery, may also be included.

The peptides and polypeptides of the invention may also be delivered with an adjuvant. Adjuvants include, but are not limited to, complete or incomplete Freund's adjuvant, Montanide ISA-51, Activation Gene-3 (LAG-3), aluminum phosphate, aluminum hydroxide, alum, and saponin. Adjuvant effects can also be obtained by injecting a variety of  
10 cytokines along with the immunogens of the invention. These cytokines include, but are not limited to IL-1, IL-2, IL-7, IL-12, and GM-CSF.

The peptides and polypeptides of the invention can also be added to professional antigen presenting cells such as dendritic cells that have been prepared ex vivo. For example, the dendritic cells could be prepared from CD34 positive stem cells from the bone marrow, or  
15 they could be prepared from CD14 positive monocytes obtained from the peripheral blood. The dendritic cells are generated ex vivo using cytokines such as GM-CSF, IL-3, IL-4, TNF, and SCF. The cultured DC are then pulsed with peptides at various concentrations using standard methods that are well known in the art. The peptide-pulsed dendritic cells can then be administered either intravenously, subcutaneously, or intradermally, and the immunization  
20 may also include cytokines such as IL-2 or IL-12.

The present invention is also directed to a vaccine in which an immunogen of the present invention is delivered or administered in the form of a polynucleotide encoding the a polypeptide or active fragment as disclosed herein, whereby the peptide or polypeptide or active fragment is produced in vivo. The polynucleotide may be included in a suitable  
25 expression vector and combined with a pharmaceutically acceptable carrier. For example, the peptides or polypeptides could be expressed in plasmid DNA and nonreplicative viral vectors such as vaccinia, fowlpox, Venezuelan equine encephalitis virus, adenovirus, or other RNA or DNA viruses. These examples are meant to be illustrative only and should not be viewed as self-limiting. A wide variety of other vectors is available and are apparent to those skilled  
30 in the art from the description given herein. In this approach, a portion of the nucleotide sequence of the viral vector is engineered to express the peptides or polypeptides of the invention. Vaccinia vectors and methods useful in immunization protocols are described in U.S. Pat. No. 4,722,848, the disclosure of which is incorporated herein by reference in its entirety.

Regardless of the nature of the composition given, additional therapeutic agents may also accompany the immunogens of the present invention. Thus, for purposes of treating tumors, compositions containing the immunogens disclosed herein may, in addition, contain other antitumor pharmaceuticals. The use of such compositions with multiple active ingredients is left to the discretion of the clinician.

In addition, the immunogens of the present invention can be used to stimulate the production of antibodies for use in passive immunotherapy, for use as diagnostic reagents, and for use as reagents in other processes such as affinity chromatography.

The present invention also relates to antibodies that react with immunogens, such as a polypeptide comprising one or more of the epitopic peptides of SEQ ID NO: 1-258 as disclosed herein. Active fragments of such antibodies are also specifically contemplated. Such antibodies, and active fragments of such antibodies, for example, and Fab structure, may react with, including where it is highly selective or specific for, an immunogenic structure comprising 2, 3, 4 or more of the epitopic peptides of the invention.

With the advent of methods of molecular biology and recombinant technology, it is now possible for the artisan or ordinary skill to produce antibody molecules by recombinant means and thereby generate gene sequences that code for specific amino acid sequences found in the polypeptide structure of the antibodies. Such antibodies can be produced by either cloning the gene sequences encoding the polypeptide chains of said antibodies or by direct synthesis of said polypeptide chains, with in vitro assembly of the synthesized chains to form active tetrameric ( $H_2L_2$ ) structures with affinity for specific epitopes and antigenic determinants. This has permitted the ready production of antibodies having sequences characteristic of neutralizing antibodies from different species and sources.

Regardless of the source of the antibodies or nanobodies, or how the artisan of ordinary skill chooses to produce such antibodies or nanobodies, including recombinantly constructed or synthesized, in vitro or in vivo, by using transgenic animals, such as cows, goats and sheep, or by using cell cultures in bioreactors, or by direct chemical synthesis employing no living organisms at any stage of the process, all antibodies and nanobodies have regions capable of interacting with a structurally complementary antigenic target. The regions interacting with the target are referred to as "variable" or "V" regions and are characterized by differences in amino acid sequence from antibodies of different antigenic specificity.

The antibodies disclosed according to the invention may also be wholly synthetic, wherein the polypeptide chains of the antibodies are synthesized and, possibly, optimized for

binding to the polypeptides disclosed herein as being receptors. Such antibodies may be chimeric or humanized antibodies and may be fully tetrameric in structure, or may be dimeric and comprise only a single heavy and a single light chain. Such antibodies may also include fragments, such as Fab and F(ab<sub>2</sub>)' fragments, capable of reacting with and binding to any of the polypeptides disclosed herein as being receptors.

A further embodiment of the present invention relates to a method for inducing a CTL response in a subject comprising administering to subjects that express HLA A1, A2 or A3 supertype antigens an effective (i.e., CTL-stimulating amount) of an immunogen of the invention that does not comprise the entire protein expressing the epitopic peptides disclosed herein (i.e., one that comprises less than the entire protein where the protein is a naturally occurring polypeptide) in an amount sufficient to induce a CTL response to tumor cells expressing at least HLA-A1 or HLA-A2, as the case may be, thereby eliciting a cellular response against said tumor cells.

A still further embodiment of the present invention relates to a method for inducing a CTL response in a subject, wherein the immunogen is in the form of a polynucleotide. In one non-limiting example, the method comprises administering to subjects that express HLA-A2 at least one CTL epitope, wherein said epitope or epitopes are selected from a group comprising the peptides disclosed according to the invention, and are coded within a polynucleotide sequence that does not comprise the entire protein coding region, in an amount sufficient to induce a CTL response to tumor cells expressing HLA-A2.

While the examples are provided below to illustrate the invention, it is to be understood that these methods and examples in no way limit the invention to the embodiments described herein and that other embodiments and uses will no doubt suggest themselves to those skilled in the art. All publications, patents, and patent applications cited herein are hereby incorporated by reference, as are the references cited therein. It is also to be understood that throughout this disclosure where the singular is used, the plural may be inferred and vice versa and use of either is not to be considered limiting.

### Example 1

#### Cell Lines

MDA-mb-231 (HLA-A2, A24), a mammary gland ductal carcinoma cell line established from a pleural effusion, was obtained from ATCC (Manassas, VA) and cultured according to the ATCC protocol. The cell line SKOV3.A2 is an HLA-A2.1 transfectant of the original ATCC (Manassas, VA) ovarian adenocarcinoma line SKOV3 (HLA-A3, 68, B18, 35,



Cw5, —) and was obtained from Dr Constantin Ioannides (M. D. Anderson Cancer Center, Houston, TX). A second ovarian cancer cell line OVCAR3 (HLA-A2, 29 B7, 58) was procured from ATCC. Both cell lines were cultured according to methods described in Ramakrishna, V. et al. 2003 International Immunology 15(6):751-763.

5

## Example 2

### Immunoaffinity Purification

All tumor lines were maintained in RPMI 1640 medium containing 10% heat-inactivated FBS, 2 mM L-glutamine, 10 mM HEPES, penicillin (100 U/ml)—streptomycin (50  
10  $\mu\text{g/ml}$ ) solution and 1% sodium pyruvate solution (all from Sigma, St Louis, MO). The SKOV3.A2 cell line was continuously maintained in 250 $\mu\text{g/ml}$  G418 (Invitrogen). The cells were harvested by treatment with 0.45% trypsin and 0.32 mM EDTA, washed two times in phosphate-buffered saline solution (pH 7.4), and stored as cell pellets at  $-80^{\circ}\text{C}$ . Aliquots of  $6-8 \times 10^{10}$  cells were solubilized at  $5-10 \times 10^6$  cells/ml in 20 mM Tris, pH 8.0, 150 mM  
15 NaCl, 1% CHAPS, 18.5  $\mu\text{g/ml}$  iodoacetamide, 5  $\mu\text{g/ml}$  aprotinin, 10  $\mu\text{g/ml}$  leupeptin, 10  $\mu\text{g/ml}$  pepstatin A, 5 mM EDTA, 0.2% sodium azide, and 17.4  $\mu\text{g/ml}$  phenylmethylsulfonyl fluoride for 1 h. This and all subsequent steps were performed with ice-cold solutions and at  $4^{\circ}\text{C}$ . The lysates were then centrifuged at 100,000 X g, the pellets discarded, and the supernatants passed through a 0.22  $\mu\text{m}$  filter. The supernatants were then passed over a series  
20 of columns with the first containing Sepharose, and the second containing the HLA-A1-specific monoclonal antibody, GAP-A1, bound to a protein A-Sepharose matrix. The second column was then sequentially washed with 20 column volumes of 20 mM Tris, pH 8.0, 150 mM NaCl, 20 column volumes of 20 mM Tris, pH 8.0, 1.0 M NaCl, and 20 column volumes of 20 mM Tris, pH 8.0. The peptides were eluted from the column with 5 column volumes of  
25 10% acetic acid. The isolated HLA-A1 molecules were then boiled for 5 min to further dissociate any bound peptide from the heavy chains. The peptides were then separated from the co-purifying class I heavy chain and  $\beta_2$ -microglobulin by centrifugation on a Ultrafree-CL membrane with a nominal molecular weight cut-off of 5,000 Daltons (Millipore, Bedford, Mass.).

30 OVCAR3 or SKOV3 cells were prepared using the same procedure as just described except that HLA-A2 molecules were prepared using HLA-A2-specific antibodies.

## Example 3

### Peptide Fractionation

The peptide extracts were fractionated by RP-HPLC (Reversed Phase -High Performance Liquid Chromatography) using an Applied Biosystems (ABI) model 140B system. The extracts were concentrated by vacuum centrifugation from about 20 ml down to 250  $\mu$ l and injected into either a Brownlee (Norwalk, Conn.) C<sub>18</sub> Aquapore column (2.1 mm X 3 cm; 300 Å; 7  $\mu$ m) or a Higgins (Mountain View, Calif.) C18 Haisil column (2.1 mm X 4 cm; 300 Å; 5 $\mu$ m). The peptides were eluted by first using a gradient of acetonitrile/0.085% TFA (trifluoroacetic acid) in 0.1% TFA/water, with the concentration of acetonitrile increasing from 0-9% (0-5 minutes), 9-36% (5-55 minutes), and 36-60% (55-62 minutes). A second dimension fractionation of combined fractions 17 and 18 from the first dimension (TFA) fraction was accomplished using the same gradient but with the substitution of HFBA (heptafluorobutyric acid) for TFA. The flow rate was 200  $\mu$ l/min, and fractions were collected at 1 min (Brownlee column) or 40 second (Higgins column) intervals. A third dimension of RP-HPLC was achieved using an Eldex (Napa, Calif.) MicroPro Pump, a homemade C<sub>18</sub> microcapillary column, and an ABI model 785A UV absorbance detector. The column was prepared by packing a 27 cm bed of 10  $\mu$ m C<sub>18</sub> particles in a section of 285  $\mu$ m o.d./75  $\mu$ m i.d. fused silica (Polymicro Technologies, Phoenix, Ariz.). Peptides in combined fractions 26 and 27 of the second dimension fraction were loaded onto this column and eluted with a gradient of acetonitrile/0.67% triethylamine acetate/water in 0.1% triethylamine acetate/water, with the concentration of acetonitrile increasing from 0-60% in 40 minutes. The flow rate was about 300 nl/min, and fractions were collected into 25  $\mu$ l of water every 30 sec. In all RP-HPLC experiments, peptides were detected by monitoring UV absorbance at 214 nm.

#### Example 4

##### Mass Spectrometric Analysis

The second dimension HPLC fraction was analyzed using an affluent splitter on the microcapillary HPLC column. In this experiment, the column (360  $\mu$ m o.d. X 100  $\mu$ m i.d. with a 25 cm C<sub>18</sub> bed) was butt connected with a zero dead volume tee (Valco, Houston, TX.) to two pieces of fused silica of different lengths (25  $\mu$ m and 40  $\mu$ m i.d.). Peptides were eluted with a 34 min gradient of 0-60% acetonitrile. The 25  $\mu$ m capillary deposited one-fifth of the HPLC effluent into the wells of a microtiter plate for use in CTL epitope reconstitution assays, whereas the remaining four-fifths of the effluent was directed into the mass spectrometer. Ions were formed by electrospray ionization, and mass spectra were recorded by scanning between mass to charge ratios (m/z) 300 and 1400 every 1.5 seconds. Peptide

sequences were determined by CAD (collision-activated dissociation) tandem mass spectrometry as described in the literature (Hunt, D. F. et al., Proc. Natl. Acad. Sci. U.S.A., 83:6233-6237, (1986)).

## 5 Example 5

### Homology searches of identified peptide sequences

Proteins containing peptides corresponding to the masses identified by MS were analyzed with the search algorithm, SEQUEST. Searches were carried using SwissProt, a curated human protein database <http://www.expasy.org/sprot/>. Table 2 describes SEQ ID  
10 NO: 1-258, which are MHC-associated peptides (active fragments) isolated from MDA-mb-231 tumor cells. Table 3 describes SEQ ID NO: 259-486, which are MHC-associated peptides (active fragments) found in one or more of the tumor cell lines MDA-mb-231 (M), OVCAR3 (O) and SKOV3.A2 (S). These tables illustrate peptides that are associated with HLA molecules, and the genes and proteins from which these peptides are derived. The tables  
15 illustrate that more than one peptide associated with HLA molecules may be derived from a single parent protein. Furthermore, many peptides and parent proteins are common to more than once tumor cell source, illustrating the shared nature of HLA-associated peptides among different tumor types.

## 20 Example 6

### Peptide Synthesis

Peptides were synthesized using a Gilson (Madison, Wis.) AMS 422 multiple peptide synthesizer. Quantities of 10  $\mu$ Mol were synthesized using conventional Fmoc amino acids, resins, and chemical techniques. Peptides were purified by RP-HPLC using a 4.6 mm X 100  
25 mm POROS (Perseptive Biosystems, Cambridge, Mass.) column and a 10 min, 0-60% acetonitrile in 0.1% TFA gradient.

## Example 7

### Generation of monocyte-derived DC and peptide loading

30 PBMC were purified from HLA-A2<sup>+</sup> normal donor blood using lymphocyte separation media (Cappel ICN Biomedical, Aurora, OH). PBMC ( $5.3 \times 10^6$ ) were added to individual wells of a 24-well cluster plate (Costar, Corning, NY) in 1.0 ml of serum-free AIM-V medium (Life Technologies) and allowed to adhere for 60 min at 37°C. Non-adherent cells were removed and saved as a source of effector T cells. Adherent PBMC ( $\sim 8.3 \times$

10<sup>5</sup>/well) were then pulsed with 50 mg/ml synthetic peptides in serum-free AIM-V medium containing 1.5 mg/ml  $\beta_2$ -microglobulin (Calbiochem-Novabiochem, San Diego, CA) and incubated for 2 h at 37°C. Unbound peptides were aspirated and the wells washed with media.

5 Monocyte-derived DC were generated as follows. PBMC (5.3 X 10<sup>7</sup>) were allowed to adhere in T-75 flasks (Corning) in 10 ml of serum-free AIM-V medium for 60 min at 37°C. Non-adherent cells were collected as a source of effector T cells and pooled with the previous collection above. Adherent monocytes in flasks were then exposed to recombinant human granulocyte macrophage colony stimulating factor (GM-CSF, 25 ng/ml; Peprotech) and recombinant human IL-4 (100 ng/ml; Peprotech) in 10 ml of AIM-V medium containing  
10 10% heat-inactivated FBS. DC obtained by this method [immature DC (iDC)] are characterized by expression of low levels of CD83, CD80, CD86, and HLA class I and class II molecules (data not shown).

Mature DC (mDC) were obtained by exposing day 5 DC cultures to recombinant  
15 soluble CD40 ligand (sCD40L; Peprotech) at 1.5 mg/ml for 24 h in the presence of 25 ng/ml GM-CSF and are characterized by expression of high levels of CD80, CD86, and HLA class I and class II molecules. mDC were harvested, washed, pulsed with 5 mg/ml peptide in serum-free AIM-V medium and irradiated (5000 rad) prior to use as stimulators.

## 20 Example 8

### Generation of peptide-specific CTL

The protocol used here is a modification of the method described by Plebanski et al. (Eur. J. Immunol. 25:1783, (1995)). CTL to peptide were generated by 3±4 cycles of stimulation with peptide-loaded APC. For the first round of stimulation (day 0), T cells or  
25 non-adherent PBMC from above (2.3 X 10<sup>6</sup>/ml or 4.3 X 10<sup>6</sup> per well) were added in bulk (CD4<sup>+</sup>, CD8<sup>+</sup>, NK, etc.) to adherent PBMC-loaded peptides in serum-free medium (50 mg/ml),  $\beta_2$ -microglobulin (1.5 mg/ml) (Calbiochem-Novabiochem), recombinant human IL-7 (5 ng/ml) (Peprotech) and keyhole limpet hemocyanin (5 mg/ml) (Sigma, St Louis, MO). Cultures were re-stimulated with iDC every 7 days, pulsed with varying amounts of  
30 peptide (second round 25 mg/ml, third round 10 mg/ml) and irradiated (5000 rad) on day 8. At each re-stimulation, the T cells were transferred to new plates by first aspirating 70% of spent media in wells and then transferring the pooled contents to a new plate. Fresh IL-7 was added at each re-stimulation. The responder:stimulator (T cell:DC) ratio was set at 20 for each

stimulation. Recombinant human IL-2 (10 U/ml) was added on day 5 after each re-stimulation.

Prior to  $^{51}\text{Cr}$ -release assay, the T cells were harvested and  $\text{CD8}^+$  T cells were purified by positive selection using  $\text{CD8}^+$  microbeads immunomagnetic cell separation with MACS kit (Miltenyi Biotec, Auburn, CA). If a fourth round of stimulation was necessary following CTL analysis, the CTL were pulsed as before, except with  $5\pm 10$  mg/ml of peptide.

### Example 9

#### Generation of allospecific CTL

HLA-A2-allospecific CTL were obtained in a mixed lymphocyte reaction by repeated stimulation of HLA-A3 $^+$  PBMC (responders) with irradiated HLA-A2 $^+$  stimulator PBMC at a ratio of 10:1 in the presence of 10 U/ml IL-2. Stimulation was repeated weekly with PBMC from different HLA-A2 $^+$  donors so as to minimize alloresponse to non-HLA-A2 antigens. T cells were assessed for lysis on several HLA-A2 $^+$  targets including tumor cells, EBV-B cells and HLA-A3 $^+$  targets every week after the third round of stimulation.

### Example 10

#### CTL expansion

Expansion of large numbers of peptide-specific or HLA-A2-allospecific CTL was achieved by culturing  $5.3 \times 10^4 \pm 1.3 \times 10^5$  T cells around day 6 or 7 post peptide- or allostimulation in the presence of  $2.5\text{--}3.0 \times 10^7$  irradiated (5000 rad) allogeneic normal donor PBMC coated with anti-CD3 antibody at 10 ng/ml (BD PharMingen, San Diego, CA) and 25 U/ml of recombinant human IL-2 (Peprotech) in a final volume of 30 ml RPMI medium. Media changes with IL-2 addition (50 U/ml) were effected on days 5 and 8. Cells were harvested for cytotoxicity assays on days 10 $\pm$ 12 and re-stimulated or frozen for later use.

### Example 11

#### $^{51}\text{Cr}$ -release cytotoxicity assay

The standard 4-h Cr-release assay was performed in 96-well V-bottomed microplates. Target cells in suspension (T2, C1R.A2, B-LCL and K562) were labeled with 100 mCi  $\text{Na}_2^{51}\text{CrO}_4$  (NEN Life Science, Boston, MA) per  $1.3 \times 10^6$  cells either overnight ( $\sim 6\pm 18$  h) in 5 ml RPMI 1640 media containing  $2\pm 5\%$  FBS or for  $60\pm 90$  min at  $37^\circ\text{C}$  directly with the cell pellet in the case of adherent cells (tumor cell lines and control lines). Labeling was

terminated by washing the targets with cold media containing 5% FBS for a total of three washes. Target cells were resuspended at a concentration of  $2-3 \times 10^4$ /ml. About  $2-3 \times 10^3$  targets in 100 ml were delivered to each well containing CTL (effectors) seeded at different E:T ratios. Spontaneous release wells contained targets in media alone, while maximal release wells contained targets in 2% NP-40 detergent (Igepal CA-630; Sigma). HLA restriction of CTL-mediated killing was achieved by preincubation of targets with HLA-specific antibodies prior to incubation with CTL.

The plate was gently spun for  $1 \pm 2$  min and incubated at  $37^\circ\text{C}$  for 4 h. For harvesting assay plates, 100 ml of supernatants from the wells was transferred to counting tubes (USA Scientific) and g counts were determined in a g counter (ICN Micromedex Systems, Huntsville, AL). Cytolytic activity of T cells was expressed in percent specific lysis as follows: specific lysis =  $\{[\text{experimental release (c.p.m.)} \pm \text{spontaneous release (c.p.m.)}] / [\text{maximal release (c.p.m.)} \pm \text{spontaneous release (c.p.m.)}]\}$ .

### Example 12

#### Competitive inhibition assay

Peptide-stimulated CTL were reacted with  $^{51}\text{Cr}$ -labeled Ov2 tumor cells (E:T ratio of 40) in the presence of excess of cold targets in a 4-h Cr-release assay. Cold targets were either empty T2 cells, T2 cells pulsed with 1 mg/ml relevant peptide (used to stimulate CTL) or irrelevant (control) peptides (HER-2/neu 369±377 or MART 127±35), or IFN- $\gamma$  pre-treated tumor cells (SKOV3.A2 and OVCAR 3) with the cold target in 5-fold excess of the hot target. Results indicate that (i) CTL show specific interaction with the peptide to which they are sensitized to, and (ii) CTL compete for similar epitopes presented on Ov2 as were found in SKOV3.A2 and OVCAR3 cell lines by MS.

Table 2. Description of Fragments, Parent Sequence Identification, Parent SwissProt Identification Number for Peptides 1-258.

SEQ ID NO:	Peptide Fragment	Parent Sequence Identification	SwissProt ID No.
1	KLLWPELLL	40S ribosomal protein S16	P62249
2	RLLDSEIKI	26S protease regulatory subunit 6A	P17980
3	KLDGSRLIKV	40S ribosomal protein S7 (S8)	/:spt[P23821]

4	RLQTQVFKL	40S ribosomal protein S9	/:spt[P46781]
5	YLGRLAHEV	60S ribosomal protein L13a (23 kDa highly basic protein)	/:spt[P40429]
6	MEHIHKLKA	60S ribosomal protein L19	/:spt[P84098]
7	KLYDIDVAKV	60S ribosomal protein L23a	/:spt[P62750]
8	IKRKNKHGW	60S ribosomal protein L34, mitochondrial precursor	/:spt[Q9BQ48]
9	DTMDHYRTF	Acidic fibroblast growth factor intracellular binding protein	/:spt[Q43427]
10	KMADIEYRL	Activator 1 36 kDa subunit	/:spt[P40937]
11	ENEFLLLL	Active breakpoint cluster region-related protein	/:spt[Q12979]
12	EPEREVVVPI	ADAMTS-15	Q8TE58
13	FLLDGFPRTV	Adenylate kinase isoenzyme 2, mitochondrial	/:spt[P54819]
14	VDSVVITESD	A-kinase anchor protein 9	/:spt[Q99996]
15	KMNEKLYTV	Androgen-induced prostate proliferative shutoff associated protein	/:trm[Q9Y451]
16	SLAEKIQAL	Ankyrin repeat and SOCS box containing protein 6 (ASH-6)	/:spt[Q9NWX3]
17	ALYEDRYLL	Argininosuccinate synthase	/:spt[P00966]
18	EQVFLSFAH	ATP-binding cassette, sub-family A, member 3	/:spt[Q99758]
19	KLIEKNYFL	ATP-dependent RNA helicase DDX18	Q9NVP1
20	TLGDLLEKEV	Autophagy protein 5-like (APG5-like)	/:spt[Q9H1Y0]
21	FLTRLQVHL	Baculoviral IAP repeat-containing protein 6	/:spt[Q9NR09]
22	RLLDYVATV	Baculoviral IAP repeat-containing protein 6	/:spt[Q9NR09]
23	GLDDYGARSM	BAP2-beta protein (Insulin receptor substrate p53)	/:trm[Q9UQ43]
24	SLEKSVLLEL	basic transcription factor 2	Q8IY69
25	ALLNQHYQV	Bifunctional aminoacyl-tRNA synthetase	/:spt[P07814]
26	ILDSGKIVQI	Bifunctional aminoacyl-tRNA synthetase	/:spt[P07814]
27	KLLETELLQEI	B-lymphoma- and BAL-associated protein	/:spt[Q8TDB6]
28	KLMEEIREL	BM-005	Q9NZF0
29	GLWEKSKGDINL	Bone morphogenetic protein receptor type II precursor	/:spt[Q13873]
30	YLINEIDRIRA	Bullous pemphigoid antigen 1, isoforms 6/9/10	/:spt[Q94833]
31	RAIDFSPYL	CCAAT/enhancer binding protein beta (C/EBP beta)	/:spt[P17676]
32	LLIDDKGTIKL	Cell division control protein 2 homolog p34protein kinase	P06493
33	KLADFGLARA	Cell division protein kinase 2	/:spt[P24941]
34	NELEKICEI	CENP-F kinetochore protein (Centromere protein F) (Mitotin)	/:spt[P49454]
35	PLSLGKENLA	CENP-F kinetochore protein (Centromere protein F) (Mitotin)	/:spt[P49454]
36	NLNOFLPEL	Centriole associated protein CEP110	/:trm[Q9Y489]
37	RLMEDVEAEKL	Cerebral cell adhesion molecule	/:trm[Q9UMW5]
38	SNRVKDLMV	Cisplatin resistance related protein CRR9p	/:trm[Q96KA5]
39	FDVQFLGSVE	C-jun-amino-terminal kinase interacting protein 2	/:spt[Q13387]
40	NYDKVEKLF	Cleavage stimulation factor 77kDa subunit	/:trm[Q12996]
41	ALILEPSLYTV	Coatamer zeta-1 subunit HSPC181	/:trm[Q9UHY0]
42	SLLOKQIML	Cohen syndrome 1 protein	/:trm[Q7Z7G8]
43	HLGVKVFVS	Collagen alpha 1(VI) chain precursor	/:spt[P12109]

44	LHELVIKTL	Colon cancer-associated protein Mic1	/:trm Q9Y5M0
45	GLVDEKALAQA	C-terminal binding protein 2	P56545
46	VMLEYVERA	Cullin homolog 2	/:spt Q13617
47	KLYTGLREV	Cullin homolog 3	/:spt Q13618
48	SLHTELNSV	Cullin homolog 7 (CUL-7)	/:spt Q14999
49	SLIDADPYL	Cyclin A2 (Cyclin A)	/:spt P20248
50	QILEYELL	Cyclin H (MO15-associated protein)	/:spt P51946
51	FLIENDLLK	Cytohesin 1 (SEC7 homolog B2-1)	Q15438
52	FLIENDLLQ	Cytohesin 3 (ARF nucleotide-binding site opener 3)	/:spt O43739
53	TEVDKILKV	DEAD box protein	/:trm Q9H4E3
54	SPLEDLSFPCA	Death associated transcription factor 1	/:spt Q9BTC0
55	KLIDKLDSL	Dedicator of cytokinesis protein 4	/:spt Q8N1I0
56	FLPTILNQL	Dedicator of cytokinesis protein 9	/:spt Q9BZ29
57	SLSSVLSHV	Dehydrogenase/reductase SDR family member 1	Q96LJ7
58	DKNATILEL	Desmoplakin (DP)	/:spt P15924
59	DLEKQIKQL	Desmoplakin (DP)	/:spt P15924
60	REYENELAKV	Desmoplakin (DP)	/:spt P15924
61	SLLEKELESV	Developmentally regulated GTP-binding protein 2 (DRG 2)	/:spt P55039
62	GLIEKNIEL	DNA (cytosine-5)-methyltransferase 1	/:spt P26358
63	YLPPEAFTF	DNA (cytosine-5)-methyltransferase 1	/:spt P26358
64	LLADLLHNV	DNA fragmentation factor 40 kDa subunit	/:spt O76075
65	AQFGYYFRV	DNA mismatch repair protein Msh2	/:spt P43246
66	LLQDFNRFL	DNA-dependent protein kinase catalytic subunit	/:spt P78527
67	LMTKEISSV	DNA-dependent protein kinase catalytic subunit	/:spt P78527
68	RLLEEALLR	DNA-dependent protein kinase catalytic subunit	/:spt P78527
69	SLLDENNVSYL	DNA-dependent protein kinase catalytic subunit	/:spt P78527
70	LLFEGEKTI	DNA-directed RNA polymerase II subunit 11	/:trm Q8TDE6
71	CLQMDKVL	Downstream neighbor of Son protein (B17)	Q9NYP3
72	ILLDERGQIKL	Dual specificity mitogen-activated protein kinase kinase 7	/:spt O14733
73	LEFDPAQRI	Dual specificity protein kinase CLK3	/:spt P49761
74	FFLDIYHNV	Dynein heavy chain, cytosolic (DYHC)	/:spt Q14204
75	ANVENVFH	dystrobrevin alpha	Q13499
76	LLAEKIYKI	E1A-associated protein p300	/:spt Q09472
77	TVLSNNLSPF	E1A-associated protein p300	/:spt Q09472
78	ALANHUKV	EH-domain containing protein 3	/:spt Q9NZN3
79	IIMEEVVKL	Envoplakin (210 kDa paraneoplastic pemphigus antigen)	/:spt Q92817
80	VEKQRPKVQ	Envoplakin (210 kDa paraneoplastic pemphigus antigen)	/:spt Q92817
81	VTSGDPELPQV	Epiregulin precursor	/:spt O14944
82	AIIEEYLHL	Eukaryotic translation initiation factor 4 gamma	/:spt Q04637
83	FLQEGDLISA	Exosome complex exonuclease RRP4	/:spt Q13868
84	MLYDTSILY	Exosome complex exonuclease RRP4	/:spt Q13868
85	YLAHFIEGL	Exportin 4 (Exp4)	/:spt Q9C0E2



86	TETVYVQDRG	Filamin A (Alpha-filamin)	
87	IQDNHDDGTYYTV	Filamin A (Alpha-filamin)	/:spt P21333
88	AIASLIGVEG	FKBP-rapamycin associated protein (FRAP)	/:spt P42345
89	GVPDDIFKL	Flightless-I protein homolog	/:spt Q13045
90	MKIKEVKKE	Frizzled-related protein	/:trm Q9BZZ6
91	YLNDLHEVL	G1/S-specific cyclin E1	/:spt P24864
92	HLAKNVVMV	G2/mitotic -specific cyclin B1	P14635
93	EALQKQLDE	GA binding protein beta-1 chain	/:spt Q06547
94	ALVDHLNVGV	GCN5-like protein 1 (RT14 protein)	P78537
95	TIABVGKWLQA	Glycogen debranching enzyme (Glycogen debrancher)	/:spt P35573
96	KLSELQLRV	Glycoprotein 25L2 precursor	/:spt Q9BVK6
97	SLFAGGMLRV	Glycoprotein 25L2 precursor	/:spt Q9BVK6
98	RLDSELKEL	Golgi autoantigen, golgin subfamily A member 3 (Golgin-160)	/:spt Q08378
99	YMDQWVPVI	Growth arrest and DNA-damage-inducible protein GADD45 alpha	/:spt P24522
100	NSLSQLEAH	Hematopoietic zinc finger protein (DKFZP586G1122 protein)	Q96PM9
101	IKGDDLVF	Heparan sulfatase 6-sulfotransferase-2	trm Q86SM9
102	RMATGLERMGA	Heterogeneous nuclear ribonucleoprotein M	/:spt P52272
103	ALIAATHIDV	High-affinity cAMP-specific and IBMX-insensitive 3',5'-cyclic phosphodiesterase 8A	/:spt Q60658
104	FMDDKRVYI	Histone deacetylase 11 (HD11)	/:spt Q96DB2
105	YLIDRVMTV	Histone deacetylase 3 (HD3) (RPD3-2)	/:spt Q15379
106	DILHQSPNM	Histone deacetylase 9 (HD9) (HD7B) (HD7)	Q9UKV0
107	NLIDLDLYV	HO complex subunit 2 (Tho2)	/:spt Q60333
108	KLTEVHEEL	Hook1 protein	/:trm Q60561
109	HLVDYTVKL	Huntingtin interacting protein 1 (HIP-1)	/:spt Q00291
110	ALLDKLYAL	Hypothetical protein (BRMS2)	/:trm Q9NV31
111	HLAEALHQA	Hypothetical protein (Fragment)	/:trm Q96CZ3
112	GLLPDVPSL	Hypothetical protein	/:trm Q9BPV2
113	IDMICAIDL	Hypothetical protein FLJ21827	/:trm Q9H6V5
114	LLIDDEYKV	Hypothetical protein KIAA0090	/:trm Q14700
115	NILPPLLQL	Importin alpha-1 subunit	/:spt P52294
116	GHEPLMNL	Importin alpha-2 subunit (Karyopherin alpha-2 subunit)	/:spt P52292
117	EFSKEPELM	Inner centromere protein	/:spt Q9NQS7
118	TLFNVKSV	Inositol 1,4,5-trisphosphate receptor type 3	/:spt Q14573
119	LMYDGTKEV	Interferon regulatory factor 6 (IRF-6)	/:spt Q14896
120	SLLSELQHA	Interferon-induced guanylate-binding protein 5	/:spt Q96PP8
121	KMVGDVTGAQA	Interferon-induced transmembrane protein 1	/:spt P13164
122	NIMDIKIGL	IQGAP3	/:trm Q86VI3
123	ALNNKLYAI	Kelch-like protein 4	/:spt Q9C0H6
124	VLDDKLYVV	Kelch-like protein 5	spt Q96PQ7
125	KLLETKWTI	Keratin, type II cytoskeletal 7 (Cytokeratin 7)	/:spt P08729
126	HLDVKPAHI	Kinase MYT1	/:trm Q99640

127	LKPDQVEGIQ	Kinectin (Kinesin receptor) (CG-1 antigen)	/:spt Q86UP2
128	SLKAHVQEV	Kinectin (Kinesin receptor) (CG-1 antigen)	/:spt Q86UP2
129	IKEAQKNAIQI	Kinesin-like protein KIF14	/:spt Q15058
130	LEDELRLQEV	Kinesin-like protein KIF14	/:spt Q15058
131	DPLMSECLL	Kinesin-like protein KIF1A	/:spt Q12756
132	YLEIYNNEEV	Kinesin-like protein KIF3A	/:spt Q9Y496
133	SLNEYEAKL	Laminin alpha-3 chain precursor (Epiligrin 170 kDa subunit)	/:spt Q16787
134	SVVPDLPVFL	Leukemia-associated protein with a CXXC domain	/:trm Q8NFU7
135	FLIHLAIH	MAGE 6	P43360
136	SLIDVNQS	Mago nashi protein homolog	/:spt P61326
137	ALFQHITAL	Medulloblastoma antigen MU-MB-50.4	/:spt Q9P055
138	VLQGLAEV	Microtubule-actin crosslinking factor 1, isoform 4	/:spt Q96PK2
139	ETELTYPTN	Microtubule-associated protein 1A (MAP 1A)	/:spt P78559
140	SLADDSVLERL	Midasin (MIDAS-containing protein)	/:spt Q9NU22
141	TLSERLWGL	Mitochondrial import receptor subunit TOM22 homolog	/:spt Q9NS69
142	YLKIFMEEV	Mitogen-activated protein kinase kinase kinase 6	/:spt O95382
143	KFSPNTSQF	Mitotic checkpoint protein BUB3	/:spt O43684
144	KIVDFSYSV	Mitotic checkpoint serine/threonine-protein kinase BUB1 beta	/:spt O60566
145	VEDKQEVNV	Mucosa associated lymphoid tissue lymphoma translocation protein 1	Q9UDY8
146	SLLEKSLGL	Multisynthetase complex auxiliary component p18	O43324
147	KIGDEPPKV	Myeloid leukemia factor 1	/:spt P58340
148	PMTLQLSLSL	myeloid/lymphoid leukemia 2	gb AAD26113.1
149	NSKITKVMLL	Myeloid/lymphoid or mixed-lineage leukemia protein 2	/:spt O14686
150	KDLEGVEVK	Myeloid/lymphoid or mixed-lineage leukemia protein 3 homolog	/:spt Q8NEZ4
151	KMPDQFDQA	Myosin X	/:spt Q9HD67
152	YLLEKNRVV	Myosin X	/:spt Q9HD67
153	LLAEKCEQL	N33 protein	/:spt Q13454
154	LDLQQNLD	NACHT-, LRR- and PYD-containing protein 2	/:spt Q9C000
155	QKSLLRSV	Nesprin 1 (Nuclear envelope spectrin repeat protein 1)	q9c0a7
156	IQTQLETLK	Nesprin 1 (Nuclear envelope spectrin repeat protein 1)	Q9C0A7
157	MDIDVPDVE	Neuroblast differentiation associated protein AHNAK	Q09666
158	YLDEQLQNL	NIMA-related kinase 11L	/:trm Q8NG66
159	EEDEDSLAD	Nuclear protein, NP220	/:trm Q14966
160	YEEDPKSKP	Nucleic acid helicase DDXx	/:trm Q8IWW2
161	NKLTLLQ	P37NB	Q13288
162	TLDDGKATL	Paired box protein Pax-8	/:spt Q06710
163	KLLGELHTL	Pericentriol material 1	Q15154
164	GLIDWNMFV	Periplakin (195 kDa cornified envelope precursor) (190 kDa p	/:spt O60437
165	SLLDLEQKL	Peroxisomal assembly protein PRX3	P56589
166	LLDHRVLEV	Plakophilin 4 (p0071)	/:spt Q99569

167	SIEEFQAK	Plectin 1 (PLTN) (PCN)	/:spt Q15149
168	SLAQYLINV	Poly(rC)-binding protein 2 (Alpha-CP2) (hnRNP-E2)	/:spt Q15366
169	LMFPINLLI	Polycystic kidney disease 1-like 2	/:trm Q7Z442
170	KLHDVEHVL	Prefoldin subunit 5	/:spt Q99471
171	ALKDLINEA	Proliferating cell nuclear antigen (PCNA) (Cyclin)	/:spt P12004
172	FLPQKHLYL	Proteasome activator complex subunit 2	/:spt Q9UL46
173	YLNQQLQE	Proteasome activator complex subunit 2	/:spt Q9UL46
174	SLIERDLKL	Protein KIAA0539	/:spt O60287
175	TVIDQIPFL	Protein phosphatase 2C delta isoform	O15297
176	KLIFLLLQL	Protein pM5 precursor	/:spt Q15155
177	FLLSLFP GAL	Protocadherin gamma B2 precursor (PCDH-gamma-B2)	/:spt Q9Y5G2
178	SLDAAAGMLYL	Proto-oncogene tyrosine-protein kinase FER	/:spt P16591
179	DSKEQFSSF	Putative Polycomb group protein ASXL1	/:spt Q8IXJ9
180	KLIPQLPTL	Rag C	/:trm Q9HB90
181	NLMDIKIGL	Ras GTPase-activating-like protein IQGAP1	/:spt P46940
182	KIFGSLAFL	Receptor protein-tyrosine kinase erbB-2 precursor	/:spt P04626
183	AENQPHQPS	Regulating synaptic membrane exocytosis protein 1	/:spt Q9HBA5
184	KILEDVVG V	Restricted expression proliferation associated protein 100	/:spt Q9ULW0
185	EGSMTVCTT	Rev interacting protein Rip-1	/:trm Q13601
186	ALQKRLDEV	Ribosome-binding protein 1 (Ribosome receptor protein)	/:spt Q9P2E9
187	RLQEELEKL	Ribosome-binding protein 1 (Ribosome receptor protein)	/:spt Q9P2E9
188	LHDEIHLL	RNA helicase	/:trm O43738
189	FLPPFP PPP	RNA-binding protein EWS (EWS oncogene)	/:spt Q01844
190	ALYNSKQLE	RSK-like protein	/:trm Q96838
191	KLQEEHQQL	Run-and FYVE-domain containing protein Rabip4R	/:trm Q96P51
192	KIAGRAVL	RuvB-like 2	/:spt Q9Y230
193	KLGLKPLEV	SART-1	/:trm O43290
194	SIIGRLLEV	Serine/threonine protein phosphatase PP1-alpha 1 catalytic subunit	/:spt P62136
195	ALDSGASLLHL	Serine/threonine-protein kinase ANKRD3	/:spt P57078
196	GEFTGW EKV	Serine/threonine-protein kinase ANKRD3	/:spt P57078
197	MDELHSLDP	Serine/threonine-protein kinase tousled-like 1	/:spt Q9UKI8
198	KILEELQKV	Serologically defined breast cancer antigen NY-BR-16	/:trm Q9H288
199	GLNDFIQKI	serum/glucocorticoid regulated kinase	O00141
200	GLIDKVNEL	Signal recognition particle 54 kDa protein (SRP54)	/:spt P61011
201	QLLTEENIP	Signal transducer and activator of transcription 2 (p113)	/:spt P52630
202	QTALLALALP	Colon carcinoma laminin-binding protein	/:spt Q9NU22
203	VSDYLRQSY	similar to hypothetical protein	rf XP_350876.1
204	FDQMVNLSI	Slit-1 protein	/:trm O75093
205	VNIFQDLQN	SLIT-3 protein (MEGF5)	/:trm O75094
206	ALLEKGITEA	SmcX protein (Xe169 protein)	/:spt P41229

207	ALLGRLAEL	SmcX protein (Xe169 protein)	/:spt P41229
208	YSEHGINI	SON protein (SON3)	/:spt Q9UPY0
209	AELEAREKL	Spectrin beta chain, brain 4	/:spt Q9NRC6
210	EAARRRLL	Spectrin beta chain, brain 4	/:spt Q9NRC6
211	SLLGDDALVQV	Splicing factor 3B subunit 3	/:spt Q15393
212	KLYGKPIRV	Splicing factor 3B subunit 4	/:spt Q15427
213	ALAEIAKAEL	Splicing factor, proline-and glutamine-rich	/:spt P23246
214	LQYKNVRPD	Superoxide dismutase [Mn], mitochondrial precursor	P04179
215	LASLVTQFNSG	Symplekin	/:spt Q92797
216	KLLDTLKKL	Syntaxin binding protein 1 (Unc-18 homolog)	/:spt P61764
217	LLDEFTTKLLA	Syntaxin binding protein 3 (UNC-18 homolog 3)	/:spt O00186
218	ALGTDAEGQKV	Syntaxin binding protein 3 (UNC-18 homolog 3)	O00186
219	FLLSGAAPLK	T lymphocyte activation antigen CD86 precursor	/:spt P42081
220	TLWNQELVI	T54 protein	Q92917
221	LSELEAALQL	Tau-tubulin kinase	/:trm Q8IWIY7
222	SLAYGGLTSPG	Tau-tubulin kinase	/:trm Q8IWIY7
223	ALWDEFNQL	T-box transcription factor TBX1 (T-box protein 1)	/:spt O43435
224	HELNNLLSK	TBP-associated factor 172 (TAF-172)	/:spt O14981
225	SLADVHIEV	TBP-associated factor 172 (TAF-172)	/:spt O14981
226	FVITVALGV	TCAM-1	Q9H3S6
227	NLMDDIERA	T-complex protein 1, theta subunit (TCP-1-theta)	/:spt P50990
228	LLWTDPRNV	Thrombospondin 3 precursor	/:spt P49746
229	AILAHLNTV	Timeless homolog	/:trm Q9UN51
230	KLAELQAQV	Transcription factor BTF3 homolog 3	/:spt Q13892
231	HLDEAIHVL	Transcription factor E2-alpha	/:spt P15923
232	SLDTQPKKV	Transcription factor E2-alpha	/:spt P15923
233	ASGNILAQNL	Transcription factor Sp4 (SPR-1)	/:spt Q02446
234	IIDPSQGLVQL	Transcription factor Sp4 (SPR-1)	/:spt Q02446
235	YMINFIHKL	Transcriptional enhancer factor TEF-1	/:spt P28347
236	DEKNHHEL	Transducin-like enhancer protein 3 (ESG3)	/:spt Q04726
237	KLVEFDLGA	Transforming acidic coiled-coil-containing protein 3	/:spt Q9Y6A5
238	SHLDEVWIK	Translation initiation factor IF-2, mitochondrial precursor	P46199
239	SLAGLGAYQL	Transmembrane protein 14B	/:spt Q9NUH8
240	YLADIFTKL	transposon-derived Buster1 transposase-like protein	/:trm Q86WW3
241	FISEIHQL	Tripartite motif protein 2	/:spt Q9C040
242	ALYDNVEKL	ts11 cell cycle control protein	P08243
243	TTFFCETGAGK	Tubulin alpha-4 chain (Alpha-tubulin 4)	/:spt P68366
244	EALDIMNR	Tuftelin-interacting protein 11 (HSPC006)	/:spt Q9UBB9
245	YMYHHPTSA	Tumor endothelial marker 7-related precursor	/:trm Q96PD9
246	GLMDNEIKV	Tumor necrosis factor receptor superfamily member 10B	/:spt O14763
247	SLIDGYRL	Tyrosine-protein kinase JAK2	/:spt O60674
248	FLQEKSPAV	ubiquitin associated protein 2-like KIAA0144	Q14157
249	FMQHDVQEL	Ubiquitin carboxyl-terminal hydrolase 7	/:spt Q93009

250	LLAEKVEEI	Ubiquitin conjugation factor E4 B	/:spt Q95155
251	RLQEDPPVGV	Ubiquitin-conjugating enzyme E2 B	/:spt P63146
252	KMFSDEILL	VELF1904	AAQ88979.1
253	SVITQVFHV	Vigilin (High density lipoprotein-binding protein)	/:spt Q00341
254	NHIELYEV	VPS10 domain-containing receptor SorCS3 precursor	/:spt Q9UPU3
255	SLQDEIQRV	WD-repeat protein 3	/:spt Q9UNX4
256	ALKKHLTSV	ZID, zinc finger protein with interaction domain	/:trn Q15916
257	MNVAGDVFN	Zinc finger protein 198	/:spt Q9UBW7
258	SKCENLSDEM	Zinc finger protein HRX (ALL-1) (Trithorax-like protein)	/:spt Q03164

Table 3. SEQ ID NO, Parent Protein Identification and SwissProt Identification Number for parent proteins SEQ ID NO: 259-486, Identified in One or More of the Tumor Cell Lines MDA-mb-231, SKOV3.A2, and OVCAR3.

SEQ ID NO:	Parent Sequence Identification	SwissProt ID No.
259	40S ribosomal protein S16	P62249
260	26S protease regulatory subunit 6A	P17980
261	40S ribosomal protein S7 (S8)	/:spt P62081
262	40S ribosomal protein S9	/:spt P46781
263	60S ribosomal protein L13a (23 kDa highly basic protein)	/:spt P40429
264	60S ribosomal protein L19	/:spt P84098
265	60S ribosomal protein L23a	/:spt P62750
266	60S ribosomal protein L34, mitochondrial precursor	/:spt Q9BQ48
267	Acidic fibroblast growth factor intracellular binding protein	/:spt O43427
268	Activator 1 36 kDa subunit	/:spt P40937
269	Active breakpoint cluster region-related protein	/:spt Q12979
270	ADAMTS-15	Q8TE58
271	Adenylate kinase isoenzyme 2, mitochondrial	/:spt P54819
272	A-kinase anchor protein 9	/:spt Q99996
273	Androgen-induced prostate proliferative shutoff associated protein	/:trm Q9Y451
274	Ankyrin repeat and SOCS box containing protein 6 (ASH-6)	/:spt Q9NWX5
275	Argininosuccinate synthase	/:spt P00966
276	ATP-binding cassette, sub-family A, member 3	/:spt Q99758
277	ATP-dependent RNA helicase DDX18	Q9NVP1
278	Autophagy protein 5-like (APG5-like)	/:spt Q9H1Y0
279	Baculoviral IAP repeat-containing protein 6	/:spt Q9NR09
280	BAP2-beta protein (Insulin receptor substrate p53)	/:trm Q9UQ43
281	basic transcription factor 2	Q8IY69
282	Bifunctional aminoacyl-tRNA synthetase	/:spt P07814
283	B-lymphoma- and BAL-associated protein	/:spt Q8TDB6
284	BM-005	Q9NZF0
285	Bone morphogenetic protein receptor type II precursor	/:spt Q13873
286	Bullous pemphigoid antigen 1, isoforms 6/9/10	/:spt Q94833
287	CCAAT/enhancer binding protein beta (C/EBP beta)	/:spt P17676
288	Cell division control protein 2 homolog p34protein kinase	P06493
289	Cell division protein kinase 2	/:spt P24941
290	CENP-F kinetochore protein (Centromere protein F) (Mitotin)	/:spt P49454
291	Centriole associated protein CEP110	/:trm Q9Y489
292	Cerebral cell adhesion molecule	/:trm Q9UMW5
293	Cisplatin resistance related protein CRR9p	/:trm Q96KA5
294	C-jun-amino-terminal kinase interacting protein 2	/:spt Q13387
295	Cleavage stimulation factor 77kDa subunit	/:trm Q12996
296	Coatomer zeta-1 subunit HSPC181	/:trm Q9UHY0
297	Cohen syndrome 1 protein	/:trm Q7Z7G8

298	Collagen alpha 1(VD) chain precursor	/:spt[P12109]
299	Colon cancer-associated protein Mic1	/:trm[Q9Y5M0]
300	Colon carcinoma laminin-binding protein	/:spt[Q9NU22]
301	C-terminal binding protein 2	P56545
302	Cullin homolog 2	/:spt[Q13617]
303	Cullin homolog 3	/:spt[Q13618]
304	Cullin homolog 7 (CUL-7)	/:spt[Q14999]
305	Cyclin A2 (Cyclin A)	/:spt[P20248]
306	Cyclin H (MO15-associated protein)	/:spt[P51946]
307	Cytohesin 1 (SEC7 homolog B2-1)	Q15438
308	Cytohesin 3 (ARF nucleotide-binding site opener 3)	/:spt[Q43739]
309	DEAD box protein	/:trm[Q9H4E3]
310	Death associated transcription factor 1	/:spt[Q9BTC0]
311	Dedicator of cytokinesis protein 4	/:spt[Q8N110]
312	Dedicator of cytokinesis protein 9	/:spt[Q9BZ29]
313	Dehydrogenase/reductase SDR family member 1	Q96LJ7
314	Desmoplakin (DP)	/:spt[P15924]
315	Developmentally regulated GTP-binding protein 2 (DRG 2)	/:spt[P55039]
316	DNA (cytosine-5)-methyltransferase 1	/:spt[P26358]
317	DNA fragmentation factor 40 kDa subunit	/:spt[Q76075]
318	DNA mismatch repair protein Msh2	/:spt[P43246]
319	DNA-dependent protein kinase catalytic subunit	/:spt[P78527]
320	DNA-directed RNA polymerase II subunit 11	/:trm[Q8TDE6]
321	Downstream neighbor of Son protein (B17)	Q9NYP3
322	Dual specificity mitogen-activated protein kinase kinase 7	/:spt[Q14733]
323	Dual specificity protein kinase CLK3	/:spt[P49761]
324	Dynein heavy chain, cytosolic (DYHC)	/:spt[Q14204]
325	dystrobrevin alpha	Q13499
326	E1A-associated protein p300	/:spt[Q09472]
327	EH-domain containing protein 3	/:spt[Q9NZN3]
328	Envoplakin (210 kDa paraneoplastic pemphigus antigen)	/:spt[Q92817]
329	Epiregulin precursor	/:spt[Q14944]
330	Eukaryotic translation initiation factor 4 gamma	/:spt[Q04637]
331	Exosome complex exonuclease RRP4	/:spt[Q13868]
332	Exportin 4 (Exp4)	/:spt[Q9C0E2]
333	Filamin A (Alpha-filamin)	/:spt[P21333]
334	FKBP-rapamycin associated protein (FRAP)	/:spt[P42345]
335	Flightless-I protein homolog	/:spt[Q13045]
336	Frizzled-related protein	/:trm[Q9BZZ6]
337	G1/S-specific cyclin E1	/:spt[P24864]
338	G2/mitotic -specific cyclin B1	P14635
339	GA binding protein beta-1 chain	/:spt[Q06547]
340	GCN5-like protein 1 (RT14 protein)	P78537
341	Glycogen debranching enzyme (Glycogen debrancher)	/:spt[P35573]
342	Glycoprotein 25L2 precursor	/:spt[Q9BVK6]

343	Golgi autoantigen, golgin subfamily A member 3 (Golgin-160)	/spt Q08378
344	Growth arrest and DNA-damage-inducible protein GADD45 alpha	/spt P24522
345	Hematopoietic zinc finger protein (DKFZP586G1122 protein)	Q96PM9
346	Heparan sulfatase 6-sulfotransferase-2	trm Q86SM9
347	Heterogeneous nuclear ribonucleoprotein M	/spt P52272
348	High-affinity cAMP-specific and IBMX-insensitive 3',5'-cyclic phosphodiesterase 8A	/spt Q60658
349	Histone deacetylase 11 (HD11)	/spt Q96DB2
350	Histone deacetylase 3 (HD3) (RPD3-2)	/spt O15379
351	Histone deacetylase 9 (HD9) (HD7B) (HD7)	Q9UKV0
352	HO complex subunit 2 (Tho2)	/spt O60333
353	Hook1 protein	/trm O60561
354	Huntingtin interacting protein 1 (HIP-1)	/spt O00291
355	Hypothetical protein	/trm Q9BPV2
356	Hypothetical protein (BRMS2)	/trm Q9NV31
357	Hypothetical protein (Fragment)	/trm Q96CZ3
358	Hypothetical protein FLJ21827	/trm Q9H6V5
359	Hypothetical protein KIAA0090	/trm Q14700
360	Importin alpha-1 subunit	/spt P52294
361	Importin alpha-2 subunit (Karyopherin alpha-2 subunit) (SRP1	/spt P52292
362	Inner centromere protein	/spt Q9NQ87
363	Inositol 1,4,5-trisphosphate receptor type 3	/spt Q14573
364	Interferon regulatory factor 6 (IRF-6)	/spt O14896
365	Interferon-induced guanylate-binding protein 5	/spt Q96PP8
366	Interferon-induced transmembrane protein 1	/spt P13164
367	IQGAP3	/trm Q86VI3
368	Kelch-like protein 4	/spt Q9C0H6
369	Kelch-like protein 5	spt Q96PQ7
370	Keratin, type II cytoskeletal 7 (Cytokeratin 7)	/spt P08729
371	Kinase MYT1	/trm Q99640
372	Kinectin (Kinesin receptor) (CG-1 antigen)	/spt Q86UP2
373	Kinesin-like protein KIF14	/spt Q15058
374	Kinesin-like protein KIF1A	/spt Q12756
375	Kinesin-like protein KIF3A	/spt Q9V496
376	Laminin alpha-3 chain precursor (Epiligrin 170 kDa subunit)	/spt Q16787
377	Leukemia-associated protein with a CXXC domain	/trm Q8NFI7
378	MAGE 6	P43360
379	Mago nashi protein homolog	/spt P61326
380	Medulloblastoma antigen MU-MB-50.4	/spt Q9P055
381	Microtubule-actin crosslinking factor 1, isoform 4	/spt Q96PK2
382	Microtubule-associated protein 1A (MAP 1A)	/spt P78559
383	Mitochondrial import receptor subunit TOM22 homolog	/spt Q9NS69
384	Mitogen-activated protein kinase kinase kinase 6	/spt Q95382
385	Mitotic checkpoint protein BUB3	/spt Q43684
386	Mitotic checkpoint serine/threonine-protein kinase BUB1 beta	/spt Q60566



387	Mucosa associated lymphoid tissue lymphoma translocation protein 1	Q9UDY8
388	Multisynthetase complex auxiliary component p18	Q43324
389	Myeloid leukemia factor 1	/:spt[P58340]
390	myeloid/lymphoid leukemia 2	gb[AAD26113.1
391	Myeloid/lymphoid or mixed-lineage leukemia protein 2	/:spt[Q14686]
392	Myeloid/lymphoid or mixed-lineage leukemia protein 3 homolog	/:spt[Q8NEZ4]
393	Myosin X	/:spt[Q9HD67]
394	N33 protein	/:spt[Q13454]
395	NACHT-, LRR- and PYD-containing protein 2	/:spt[Q9C000]
396	Nesprin 1 (Nuclear envelope spectrin repeat protein 1)	Q9c0a7
397	Neuroblast differentiation associated protein AHNAK	Q09666
398	NIMA-related kinase 11L	/:trm[Q8NG66]
399	Nuclear protein, NP220	/:trm[Q14966]
400	Nucleic acid helicase DDXx	/:trm[Q8IWW2]
401	P37NB	Q13288
402	Paired box protein Pax-8	/:spt[Q06710]
403	Pericentriol material 1	Q15154
404	Periplakin (195 kDa cornified envelope precursor)	/:spt[Q060437]
405	Peroxisomal assembly protein PEX3	P56589
406	Plakophilin 4 (p0071)	/:spt[Q99569]
407	Plectin 1 (PLTN) (PCN)	/:spt[Q15149]
408	Poly(rC)-binding protein 2 (Alpha-CP2) (hnRNP-E2)	/:spt[Q15366]
409	Polycystic kidney disease 1-like 2	/:trm[Q7Z442]
410	Prefoldin subunit 5	/:spt[Q99471]
411	Proliferating cell nuclear antigen (PCNA) (Cyclin)	/:spt[P12004]
412	Proteasome activator complex subunit 2	/:spt[Q9UL46]
413	Protein KIAA0539	/:spt[Q60287]
414	Protein phosphatase 2C delta isoform	Q15297
415	Protein pM5 precursor	/:spt[Q15155]
416	Protocadherin gamma B2 precursor (PCDH-gamma-B2)	/:spt[Q9Y5G2]
417	Proto-oncogene tyrosine-protein kinase FER	/:spt[P16591]
418	Putative Polycomb group protein ASXL1	/:spt[Q8LXJ9]
419	Rag C	/:trm[Q9HB90]
420	Ras GTPase-activating-like protein IQGAP1	/:spt[P46940]
421	Receptor protein-tyrosine kinase erbB-2 precursor	/:spt[P04626]
422	Regulating synaptic membrane exocytosis protein 1	/:spt[Q9HBA5]
423	Restricted expression proliferation associated protein 100	/:spt[Q9ULW0]
424	Rev interacting protein Rip-1	/:trm[Q13601]
425	Ribosome-binding protein 1 (Ribosome receptor protein)	/:spt[Q9P2E9]
426	RNA helicase	/:trm[Q43738]
427	RNA-binding protein EWS (EWS oncogene)	/:spt[Q01844]
428	RSK-like protein	/:trm[Q96S38]
429	Run-and FYVE-domain containing protein Rabip4R	/:trm[Q96P51]
430	RuvB-like 2	/:spt[Q9Y230]
431	SART-1	/:trm[Q43290]

432	Serine/threonine protein phosphatase PP1-alpha 1 catalytic subunit	/:spt[P62136]
433	Serine/threonine-protein kinase ANKRD3	/:spt[P57078]
434	Serine/threonine-protein kinase tousled-like 1	/:spt[Q9UK18]
435	Serologically defined breast cancer antigen NY-BR-16	/:trm[Q9H288]
436	serum/glucocorticoid regulated kinase	O00141
437	Signal recognition particle 54 kDa protein (SRP54)	/:spt[P61011]
438	Signal transducer and activator of transcription 2 (p113)	/:spt[P52630]
439	similar to hypothetical protein	rl[XP_350876.1]
440	Slit-1 protein	/:trm[Q75093]
441	SLIT-3 protein (MEGF5)	/:trm[Q75094]
442	SmcX protein (Xe169 protein)	/:spt[P41229]
443	SON protein (SON3)	/:spt[Q9UPY0]
444	Spectrin beta chain, brain 4	/:spt[Q9NRC6]
445	Splicing factor 3B subunit 3	/:spt[Q15393]
446	Splicing factor 3B subunit 4	/:spt[Q15427]
447	Splicing factor, proline-and glutamine-rich	/:spt[P23246]
448	Superoxide dismutase [Mn], mitochondrial precursor	P04179
449	Symplekin	/:spt[Q92797]
450	Syntaxin binding protein 1 (Unc-18 homolog)	/:spt[P61764]
451	Syntaxin binding protein 3 (UNC-18 homolog 3)	/:spt[O00186]
452	T lymphocyte activation antigen CD86 precursor	/:spt[P42081]
453	T54 protein	Q92917
454	Tau-tubulin kinase	/:trm[Q8IWY7]
455	T-box transcription factor TBX1 (T-box protein 1)	/:spt[O43435]
456	TBP-associated factor 172 (TAF-172)	/:spt[O14981]
457	T-complex protein 1, theta subunit (TCP-1-theta)	/:spt[P50990]
458	Thrombospondin 3 precursor	/:spt[P49746]
459	Timeless homolog	/:trm[Q9UNSI]
460	Transcription factor BTF3 homolog 3	/:spt[Q13892]
461	Transcription factor E2-alpha	/:spt[P15923]
462	Transcription factor Sp4 (SPR-1)	/:spt[Q02446]
463	Transcriptional enhancer factor TEF-1	/:spt[P28347]
464	Transducin-like enhancer protein 3 (ESG3)	/:spt[Q04726]
465	Transforming acidic coiled-coil-containing protein 3	/:spt[Q9Y6A5]
466	Translation initiation factor IF-2, mitochondrial precursor	P46199
467	Transmembrane protein 14B	/:spt[Q9NUH8]
468	Transposon-derived Buster1 transposase-like protein	/:trm[Q86WW3]
469	Tripartite motif protein 2	/:spt[Q9C040]
470	ts11 cell cycle control protein	P08243
471	Tubulin alpha-4 chain (Alpha-tubulin 4)	/:spt[P68366]
472	Tuftelin-interacting protein 11 (HSPC006)	/:spt[Q9UBB9]
473	Tumor endothelial marker 7-related precursor	/:trm[Q96PD9]
474	Tumor necrosis factor receptor superfamily member 10B	/:spt[O14763]
475	Tyrosine-protein kinase JAK2	/:spt[O60674]
476	Ubiquitin associated protein 2-like KIAA0144	Q14157

477	Ubiquitin carboxyl-terminal hydrolase 7	/:spt Q93009
478	Ubiquitin conjugation factor E4 B	/:spt Q95155
479	Ubiquitin-conjugating enzyme E2 B	/:spt P63146
480	VELF1904	gb AAQ88979.1
481	Vigilin (High density lipoprotein-binding protein)	/:spt Q00341
482	VPS10 domain-containing receptor SorCS3 precursor	/:spt Q9UPU3
483	WD-repeat protein 3	/:spt Q9UNX4
484	ZID, zinc finger protein with interaction domain	/:trm Q15916
485	Zinc finger protein 198	/:spt Q9UBW7
486	Zinc finger protein HRX (ALL-1) (Trithorax-like protein)	/:spt Q03164

What is claimed is:

1. An isolated oligopeptide or peptide comprising at least one epitopic peptide selected from the group consisting of SEQ ID NOS: 1 to 258.

5 2. The oligopeptide of claim 1 wherein said polypeptide comprises at least two of said epitopic peptides.

3. The oligopeptide of claim 1 wherein said polypeptide comprises at least three of said epitopic peptides.

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4. An oligopeptide or peptide comprising at least one epitopic peptide having at least one amino acid difference from an amino acid sequence selected from the group consisting of SEQ ID NO: 1 to 258.

15 6. The oligopeptide of Claim 4 wherein said one amino acid difference is the result of a conservative amino acid substitution.

7. The oligopeptide of claim 4 wherein said substitution is the substitution of one hydrophobic amino acid by another hydrophobic amino acid.

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8. The oligopeptide of claim 4 wherein said amino acid difference is the addition or deletion of one amino acid to or from said oligopeptide.

9. A nucleic acid comprising a polynucleotide that encodes a polypeptide of claim 1.

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10. A composition comprising an immunogen of claim 1 present in a pharmaceutically acceptable carrier and in an amount sufficient to elicit production of antibodies or cells that react with said immunogen when said immunogen is administered to an immunologically  
30 competent animal.

11. An antibody specific for an immunogen of claim 1.

12. A method for treating a subject with cancer, said cancer characterized by tumor cells expressing any class I MHC molecule, comprising administering to said subject a composition comprising

at least one polypeptide comprising an epitopic peptide comprising an amino acid  
5 sequence selected from the group consisting of SEQ ID NO: 1 to 258 in an amount sufficient to induce a CTL response to said tumor cells; or

at least one polypeptide comprising an epitopic peptide having at least one amino acid difference from an amino acid sequence selected from the group consisting of  
10 SEQ ID NO: 1 to 258 in an amount sufficient to induce a CTL response to said tumor cells.

13. A method for treating a subject with cancer, said cancer characterized by tumor cells expressing any class I MHC molecule, said method comprising administering to said subject a composition comprising a polynucleotide comprising a nucleic acid sequence encoding

15 at least one polypeptide comprising an epitopic peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1 to 258 in an amount sufficient to induce a CTL response to said tumor cells; or

at least one polypeptide comprising an epitopic peptide comprising one amino acid difference from an amino acid sequence selected from the group consisting of  
20 SEQ ID NO: 1 to 258 in an amount sufficient to induce a CTL response to said tumor cells.

14. A method for treating a subject with cancer, said cancer characterized by tumor cells expressing HLA A1, A2, or A3 supertypes, said method comprising

25 administering to said subject induced CTLs in an amount sufficient to destroy the tumor cells through direct lysis or to effect the destruction of the tumor cells indirectly through the elaboration of cytokines, said CTLs induced by a process comprising

inducing a cytotoxic T lymphocyte (CTL) *in vitro* that is specific for said  
tumor cells by contacting a precursor CTL with:

30 at least one polypeptide comprising an epitopic peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1 to 258 under

conditions that generate a CTL response to said tumor cells; or

at least one polypeptide comprising an epitopic peptide comprising one amino acid difference from an amino acid sequence selected from the group consisting of SEQ ID NO: 1 to 258 under conditions that generate a CTL response to said tumor cells.

15. A method for treating a subject with cancer, said cancer characterized by tumor cells expressing any class I MHC molecule, said method comprising

administering to said subject induced CTLs in an amount sufficient to destroy the tumor cells through direct lysis or to effect the destruction of the tumor cells indirectly through the elaboration of cytokines, said CTLs induced by a process comprising

inducing a cytotoxic T lymphocyte (CTL) *in vitro* that is specific for said tumor cells by contacting a precursor CTL with:

at least one polypeptide comprising an epitopic peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 259 to 486 under conditions that generate a CTL response to said tumor cells; or

at least one polypeptide comprising an epitopic peptide comprising one amino acid difference from an amino acid sequence selected from the group consisting of SEQ ID NO: 259 to 486 under conditions that generate a CTL response to said tumor cells.

16. A method for inducing a cytotoxic T lymphocyte (CTL) *in vitro* that is specific for a tumor cell expressing HLA A1, A2, or A3 supertypes comprising contacting a precursor CTL with:

at least one polypeptide comprising an epitopic peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1 to 258 under conditions that generate a CTL response to said tumor cells; or

at least one polypeptide comprising an epitopic peptide comprising one amino acid difference from an amino acid sequence selected from the group consisting of SEQ ID NO: 1 to 258 under conditions that generate a CTL response to said tumor cells.

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17. A process for inducing a CTL response *in vitro* that is specific for a tumor cell expressing HLA A1, A2, or A3 supertypes, said process comprising contacting a precursor CTL with a cell comprising

a polynucleotide comprising a nucleic acid sequence encoding at least one polypeptide comprising an epitopic peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1 to 258; or  
a polynucleotide comprising a nucleic acid sequence encoding at least one polypeptide comprising an epitopic peptide comprising one amino acid difference from an amino acid sequence selected from the group consisting of SEQ ID NO: 1 to 258.

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18. A method for treating a subject with cancer, said method comprising: stimulating the production of antibodies for use in passive immunotherapy, wherein said antibodies react with

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at least one polypeptide comprising an epitopic peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1 to 258; or

at least one polypeptide comprising an epitopic peptide comprising one amino acid difference from an amino acid sequence selected from the group consisting of SEQ ID NO: 1 to 258.

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19. A method for diagnosing the presence of cancer in a subject comprising obtaining a tissue sample from said subject; and detecting

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at least one polypeptide comprising an epitopic peptide comprising an amino acid sequence selected from the group consisting of SEQ ID NO: 1 to 258; or

at least one polypeptide comprising an epitopic peptide comprising one amino acid difference from an amino acid sequence selected from the group consisting of SEQ ID NO: 1 to 258;

in said sample.

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20. The method of claim 16 wherein said cancer is selected from the group consisting of breast carcinoma, ovarian carcinoma, colorectal carcinoma, lung carcinoma, and prostate carcinoma.